# CHEMISTRY and PHYSIOLOGY of the Vitamins

By

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## PREFACE TO THE REVISED REPRINT

The present volume is essentially a reprint of the "Chemistry and Physiology of the Vitamins" which was published in 1942. Although many new discoveries have been made since the manuscript for the first edition was written, practically none of these has been incorporated into the reprint. Only actual errors which have crept into the first edition and which have come to the attention of the author have been corrected. However, the "Recommended Daily Allowances" as published in January 1943 by the Food and Nutrition Board of the National Research Council have been added.

Wilmington, Del.
Spring 1945

H. R. ROSENBERG

# PREFACE TO THE 1942 EDITION

This monograph Chemistry and Physiology of the Vitamins, has a long historical background Exactly a decade ago it was the privilege of the author to be present at the meeting held by the Zurich section of the Swiss Chemical Society when Paul Karrer announced a few days prior to its publication the successful isolation of essentially pure vitamin A from fish liver oils and the establishment of the structure of this vitamin It was then that the author felt the urge to compile the available data on the chemistry and physiology of all vitamins The science of vitamins had by that time advanced to a state at which the existence of vitamins, brought into the limelight between 1906 and 1914, was no longer questioned In 1926 B C P Jansen and W F Donath had announced the isolation of pure, crystalline vitamin $B_1$ but the constitution of this vitamin remained unknown for many years to come At the same time (1926), *Pohl, Windaus, Hess, Rosenheim and Webster had recognized that the* long known ergosterol could be converted into a vitamin D by activation with ultraviolet light, but the pure vitamin D was not obtained until 1931–1932, and the correct chemical constitution was not known Thus, Karrer was the first to establish the true chemical constitution of a vitamin

In the years following Karrer's announcement, the author, while working in the laboratories of L Ruzicka and T Reichstein, had the good fortune of being able to see and observe the progress made on the establishment of the structure and the synthesis of several vitamins and hormones During that time Reichstein synthesized vitamin C, the first vitamin ever obtained by total synthesis And, Karrer, in a laboratory only a few blocks removed, worked in a dramatic race against Kuhn in Germany on vitamin B , and was the first to announce the successful synthesis of this vitamin From Ruzicka s laboratory the synthesis of the male sex hormones androsterone and testosterone, by degradation of cholesterol was announced, and Reichstein started to investigate the hormones of the adrenal cortex

The author was then tempted to plan the publication of a comprehensive volume on the chemistry and physiology of the vitamins and hormones These two classes of compounds have so much in common that a review of one of them seemed to necessitate a review of the other The extraordinary activity of research workers in all parts of the world, however has resulted in the accumulation of such an enormous amount of scientific and practical material that it became infeasible to combine a discussion of

both the vitamins and the hormones in one volume Thus, this mono graph is confined to a treatment of the vitamins exclusively

Since the author left the hospitable Swiss Laboratories, he has been connected, at some time or other, with the development of many of the vitamins known today Simultaneously, the need for an up to date pres entation of the chemistry and physiology of the vitamins became more and more recognized, and this need is felt today by everyone who desires to inform himself or others on this subject Short tabulations of the vitamins and scattered review articles do not answer the need Books written for the general public are obviously of a different category than books of a purely technical character Fortunately, there have been available in the English language a number of excellent books on the medical aspects of vitamin therapy such as The Vitamins,' a symposium published under the auspices of the American Medical Association and The Avitaminoses' by W H Eddy and G Dalldorf There is also a very satisfactory book on The Biological Standardization of the Vitamins written by K H Coward In addition there are monographs on special vitamins such as the book on Vitamin $B_1$ by R R Williams and T D Spies, Vitamin D by C I Reed H C Struck and I E Steck Vita min K by H R Butt and A M Snell and Vitamin E a symposium held under the auspices of The Food Group of the Society of Chemical Industry The author however, knows of no comprehensive treatment of the chemistry and physiology of all the vitamins

The present monograph on the Chemistry and Physiology of the Vitamins begins with the presentation of a definition of the vitamins which distinguishes this group of compounds sharply from the hormones and from other essential and non essential food constituents A new classification for compounds which have the dual character of vitamins and structural building units or suppliers of energy is introduced Each vitamin is then discussed separately emphasis being laid upon the chem istry and the physiological action of these compounds The chapters on each vitamin start with a review of the nomenclature and a tabulation of the historical development followed by a paragraph on the occurrence of the vitamin The main discussions on the chemistry and physiology follow Under the chemistry of each vitamin the procedures used for the isolation of the vitamin, the proof of the chemical constitution and the synthesis of the vitamin are reviewed separately There are special para graphs on industrial methods of preparing the vitamins and on their biogenesis The specificity of the vitamin action is treated separately The determination of the vitamins is subdivided into physical chemical

biochemical and biological methods, and is followed by a paragraph on the vitamin standards The physiology of plants and microorganisms is separated from the animal physiology which is subdivided into the metabolism of the vitamin, the physiological action and the mechanism of the vitamin action The relation of each vitamin to other vitamins to hormones, and to inorganics is presented in special paragraphs This is followed by a short review of the present day knowledge of the pathological aspects, the hypovitaminoses avitaminoses, hypervitaminoses and paravitaminoses, with a special section on clinical test methods Finally the vitamin requirements are briefly stated The book ends with a list and abstracts of vitamin patents which have issued in the United States of America, Great Britain, Germany and France arranged in a manner similar to that of the main text

The vitamins are presented according to the alphabetical order of nomenclature This arrangement follows in general the incidental discovery of the vitamins Eventually, a classification according to the function of the vitamins will probably prove to be more satisfactory, but cannot successfully be undertaken at this time due to the rather incomplete knowledge of the primary function of many members

This volume has been prepared with the idea of covering all topics of vitamin research and especially the chemistry and physiology of the vitamins In presenting this monograph to the public the hope is expressed that it may guide the student and the scholar through our present day knowledge of the field and inspire further development This is especially desirable and should be expedited as much as possible since the health and successful propagation of man are, to a considerable extent, dependent upon proper nutrition, and any advance in the knowledge of the vitamins can be utilized immediately for the benefit of mankind

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Winter 1941–1942

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## THE VITAMINS IN GENERAL

### 1 Definition of Vitamins

*Vitamins are organic compounds which are required for the normal growth and maintenance of life of animals, including man who, as a rule, are unable to synthesize these compounds by anabolic processes that are independent of environment other than air and which compounds are effective in small amounts do not furnish energy and are not utilized as building units for the structure of the organism, but are essential for the transformation of energy and for the regulation of the metabolism of structural units*

The science of nutrition classifies the alimentary constituents (according to their function) into two different groups energy and building unit providing food, on the one hand, and protective food on the other— The latter group comprises water inorganic substances and certain organic compounds among which are the vitamins The definition of vitamins as given above clearly differentiates this group of nutrients from all other food constituents Vitamins are organic compounds, and water or inorganic substances cannot be classified as vitamins Vitamins are required for the normal growth and maintenance of life of animals including man All known vitamins with the probable exception of vitamin D are synthesized by plants and, as far as is known are used by them essentially for the same purposes as by man and animals The latter, however, as a rule are unable to synthesize these compounds by anabolic processes which are independent of the environment with the exception of air In other words the inherently available mechanisms of organo synthesis in animals and man are not provided with means to produce the vitamins On the other hand those compounds which are produced anabolically (or catabolically) and which otherwise conform to the definition for vitamins as given above are classified as hormones The apparent independence of cattle for certain members of the vitamin B complex and for vitamin K is due to bacterial synthesis of these vitamins in the rumen which is not an anabolic process The production of vitamin D in the skin is not an anabolic process as defined since this process is not independent of the environ

ment but requires energy from the outside This energy consists of ultra violet light and is normally supplied by sunlight

Vitamins are compounds which are effective in small amounts and occur as traces in cells and body fluids While the maximum concentration in tissues or fluids with the exception of those which serve as storage places has not been determined for all vitamins, it is estimated that the amount of any one vitamin is less than 5 $\gamma$ per gram of dry weight [1] Vitamins do not furnish energy and are thus distinguished from the energy bearing food constituents The amount of vitamins needed is too small to account for even a fraction of the total energy required Actually, minute quantities of vitamins are burned in the organism but the energy set free by this process is infinitesimal The vitamins, furthermore, do not act as morphological or structural building units of the organism or its cells The fact that excessive amounts of most vitamins are excreted unchanged indicates that they are not utilized as suppliers of energy or as structural building units The vitamins are, however essential for the transformation of energy and for the regulation of the metabolism of structural units and are functional in systems which carry out these reactions Such systems are quite complex and are, as far as is known, enzymatic in character These enzyme systems consist of many different components, a few of which are vitamins

Vitamins are required by animals including man, as has been stated previously As long as at least one animal species is known to be unable to synthesize a particular compound that compound should be considered a vitamin provided it conforms with the definition for vitamins in other particulars Actually it cannot be expected that all animals need exactly the same nutritional elements In accordance with this thought it has for example been observed that the cockroach apparently does not need any vitamin A,[2] and it is conceivable that animals will be discovered which do not need any vitamin D Ascorbic acid, on the other hand while apparently needed by all animals, is synthesized by many of them, whereas other animals, such as the primates and the guinea pig are dependent upon an outside source of this compound Ascorbic acid is thus a vitamin according to the definition of this term

The classification of the nutritional elements which exert vitamin A activity requires special consideration Man and most animals obtain in their foods two different types of substances which bring about the same

[1] D E Green *Advances in Enzymology* 1 177 (1941)
[2] R I Bowers and C M McCay *Science* 92 291 (1940)

physiological effect Both groups belong to the same class of organic com pounds namely, the carotenoids They differ from each other in that the one group contains 40 carbon atoms in the molecule, while the other group contains about 20 carbon atoms The first group of substances is converted metabolically into the second group in the animal organism The second group has acquired the term vitamin A while the first group has been designated as provitamin A This terminology is not in strict agreement with the definition for vitamins according to which both groups of compounds should be called vitamins The term provitamin A has however been retained in this monograph in accordance with general usage in order to avoid further complication of the vitamin terminology, since different terms have to be applied to the two different types of com pounds

The definition of vitamins as given above, has not been undisputed The most severe criticism is born of the thought that the ingested essential nutrients exert no vitamin activity as such but are active only after chemi cal transformation into other compounds According to these views the ingested compounds should be called provitamins unless it is established that they do not undergo transformation in the body Nicotinamide and nicotinic acid according to this interpretation should be called provitamins, while nicotinamide containing coenzymes codehydrogenase I and II should be called vitamins Similarly vitamin $B_1$ (thiamin) and vitamin $B_2$ (riboflavin) should be called provitamins This definition of the term vitamin however does not appear desirable Compounds like nicotin amide and riboflavin are constituents of a number of different enzymes and a multitude of different vitamins would have to be postulated As a re sult it would be difficult in many cases to decide if the coenzyme or the entire enzyme system should be called a vitamin The term vitamin would be dissociated from the science of nutrition which needs a term for these compounds The term vitamin as defined on page 3 has served a useful purpose and it appears more logical to adopt a special term for enzymes which contain vitamins such as, for example the term vita zyme than to change radically the present day vitamin definition which has been adopted generally

There is another group of organic compounds which are protective foods They like the vitamins are required for normal growth and maintenance of life of animals including man who as a rule, are unable to synthesize these compounds by anabolic processes They are also essential for the transformation of energy and for the regulation of the metabolism of struc tural units These compounds differ from the vitamins in that they also

act as suppliers of energy or as structural building units In view of the similarity of these compounds to the vitamins but in recognition of the fact that they differ in one important functional aspect from them it is suggested that this class of compounds be called *vitagens* This term is broad and emphasizes that the compounds of this class are concerned with the production and maintenance of life, It is recommended that this terminology be adopted until the time when more precise information is available concerning the physiological action of these compounds and the vitamins

The number of compounds which should be classified as vitagens is unknown There is ample evidence that they include the essential fatty acids and the essential amino acids There may be some essential carbohydrates which conform to the vitagen concept Choline and related compounds which supply the essential *transferable methyl group are* vitagens and it is suspected that some organic sulfur containing compounds will be discovered which belong to this group The actual vitagen nature of all these compounds however, has not been demonstrated The essential fatty acids are structural units in many phospholipids and their physiological action appears to be primarily a regulatory one Some of the essential amino acids have been shown to be structural building units for tissue and cellular and intracellular fluid constituents but are also constituents of enzyme systems involved in the metabolism of energy bearing foods For example the essential amino acids lysine, histidine tryptophane, phenyl alanine and arginine are constituents[3] of the apoenzyme of a riboflavin enzyme system, and the possibility remains that some other essential amino acid is present in this system Choline and other compounds which provide an essential transferable methyl group for the anabolism of the structure of the organism also act as regulators, for example, in the distribution of fat

A dietary deficiency of any one of the vitagens gives rise to specific clinical symptoms which are similar to those encountered in vitamin deficiencies Obviously, they might be considerably complex since the vitagens have the double function of providing energy or building units on the one hand, and of being concerned with the transformation of energy or the regulation of the metabolism of structural units on the other hand A deficiency of any one of the essential amino acids which are constituents of a riboflavin enzyme system should eventually cause the occurrence of the same syndromes which are observed during times of riboflavin defi

[3] R Kuhn and P Desnuelle *Ber* 70 1907 (1937)

ciency Whether or not that is actually the case cannot be stated It might be expected that during times of a vitagen deficiency the vitagen would be liberated in the organism from the structural materials and utilized functionally This however, does not seem to be the case Thus a deficiency of choline in the diet becomes apparent from a disturbance of the distribution of fat in the liver in spite of the presence of very large amounts of choline in the phospholipids of the body [4]

## 2 History of the Discovery of the Vitamins[5]

Generally speaking three distinctly different periods in the history of vitamins can be differentiated First there was the period which is characterized by the recognition of the existence of nutrient materials different from those which are needed only for the maintenance of the energy and building unit supply This period actually began many centuries ago but became a definite science at the turn of this century The second period is that of the isolation of a great number of vitamins in pure form and the elucidation of their chemical structure which culminated in the synthesis of various vitamin compounds This period started about in the middle of the 1920 s when the first vitamins were obtained in crystalline form The last period is characterized by the recognition that various compounds which were previously known to exert beneficial effects in the growth of lower organisms such as yeast and bacteria are also necessary for man and animals Simultaneously an earnest effort is being made to understand the physiology and the mechanism of the vitamin action which has resulted so far in the recognition of the part which various vitamins, especially those of the B complex play in a number of different enzyme systems

Diseases of the human organism caused by a deficiency of vitamins are probably as old as the human race Among the uncovered skeletons of prehistoric man are some which show definite signs of rickets (vitamin D deficiency) and of scurvy (vitamin C deficiency) The symptoms of these diseases and of beriberi (vitamin $B_1$ deficiency) and of night blindness (vitamin A deficiency) were known to the physicians of ancient days and were described in various manuscripts which were written in the first thousand years A D With the exception of a remedy for night blindness no effective therapeutic methods were known at that time for curing or pre

[4] F X. Aylward H J Channon and H Wilkinson *Biochem J* 29 169 (1935)

[5] For more details of the historical development see H C. Sherman and S L. Smith *The Vitamins* New York 1931 Medical Research Council *Vitamins A Survey of Present Knowledge* London 193? v Brunn *Münch Med Wochschr* 84 223 (1937)

tional constituents He carried out experiments on rats by supplying them with a diet consisting of purified carbohydrates, fats, proteins, inorganic material and water The animals did not flourish, but growth resulted when "astonishingly" small amounts of milk were added to the diet (similar to the earlier but less exact experiments of Lunin) In place of milk, the alcohol or ether soluble fraction of milk was found to bring about the same effect In 1909 Stepp observed that similar growth promoting substances are present in bread Pekelharing had already emphasized in 1905 in a paper which was overlooked for many years that only very small amounts of these substances present in traces in milk and probably in all sorts of foodstuffs, both of vegetable and animal origin, are necessary to keep mice alive

Funk in 1912 reviewed the then existing knowledge of the diseases caused by nutritional errors They were beriberi and scurvy and possibly also rickets, sprue and pellagra Funk was apparently the first to recognize pellagra as a nutritional deficiency disease The etiology of sprue is still unknown today Funk attempted to isolate the compound which prevents beriberi and concluded that it is, chemically speaking, an amine Funk did not succeed in isolating the pure substance but obtained nicotinic acid as a by product which he showed to have some slight beneficial growth effect (Today we recognize nicotinic acid as the pellagra preventive compound ) In systematizing the knowledge of the nutritional elements other than carbohydrates, fats, proteins inorganics and water Funk called this new class of compounds "vitamines" a term which was later changed to "vitamins" (see under Nomenclature of the vitamins, page 12) Thus the first period of the history of vitamins in which the existence of the vitamins was recognized and proved is concluded

In the second period of vitamin research various vitamins were isolated in the pure state, their chemical constitution established and their chemical synthesis achieved This development was made possible by using experimental animals as tools in order to determine the presence and concentration of vitamins and for studying and differentiating the symptoms caused by special diets deficient in various constituents The isolation, the determination of the chemical structure and the synthesis of the vitamins will be discussed later At this point the historic development leads to a special history of each single vitamin This will be presented in the discussion of each vitamin under "Chronology" In this period the following vitamins were elucidated vitamin A (the compound which prevents night blindness), vitamin $B_1$ (the anti beriberi vitamin), vitamin $B_2$ vitamin $B_6$, vitamin C (the anti scurvy substance), vitamin D (the anti rickets com

pound), vitamin E and vitamin K The existence of other vitamins was postulated during this period but general knowledge did not proceed further than the recognition of the existence of further vitamins and the determination of more or less specific deficiency symptoms

The third period in the history of vitamin research proceeds along somewhat different lines of thought While in the second period the vitamin was isolated by tedious concentration procedures using the experimental animal as the tool for the determination of the progress made, a different course was followed in the next period This originated as a development of the study of the nutritional elements necessary for microorganisms, especially yeast and certain bacteria J von Liebig already had observed in the last century that yeast cannot grow properly unless the culture medium contains, besides the known and accepted nutritional elements some growth stimulating material which was found to be present in meat extracts This unknown substance was later called bios and proved to be a mixture of substances The first compound isolated from bios concentrates was inositol (1928) which Woolley identified in 1940 as a vitamin Elvehjem found that the bios factor nicotinic acid cured blacktongue, a deficiency disease of dogs This was soon followed by the isolation of nicotinic acid from crude concentrates of the anti blacktongue factor Almost immediately the effectiveness of nicotinic acid in the cure of human pellagra was discovered Several other vitamins were discovered in the same way *The yeast growth factor, pantothenic acid, proved to be the* chicken antidermatitis vitamin biotin was found to be identical with the postulated vitamin H and *p* amino benzoic acid the growth factor for *Clostridium acetobutylicum* was shown to exert vitamin activity for man and animals This development is not necessarily terminated as yet since further unknown growth factors for microorganisms apparently exist which may prove to be identical with vitamins for the animal organism

As in the case of other natural sciences the first step in vitamin research is to recognize the various members The next step is to study the physiological action and the final step is to elucidate the mechanism of the action It seems that the main task of the recognition of vitamins has been essentially accomplished No doubt the list of vitamins is not complete as yet and considerably more work needs to be done before all the vitamins will be recognized Nevertheless it appears that those vitamins which cause the most obvious deficiency diseases are known While Lunin in 1881 and Hopkins in 1906 failed to keep animals alive on a diet composed of the then known nutritional elements the situation is quite different today It has been possible in a number of different laboratories to raise experimental

animals, rats for example on a completely synthetic diet, which includes all the known vitamins, and to keep the animals alive over a period of several generations Simultaneously essential work is in progress for clarifying the physiological behavior of the vitamins in the organism Once the reactions which are carried out by the vitamins in the organism are known attempts can be made to clarify the mechanism of this action The gross physiological effect of each vitamin is evident from the symptoms caused by the deficiency of the vitamin But these clinical symptoms may be of secondary nature and the elucidation of the primary reaction is quite difficult and requires new and special technics (see under Physiology of the vitamins page 26) This work has shown that the vitamins are essential participants in the metabolism of energy bearing food of minerals and of water The *proof is in some cases a direct one in other cases indirect* and needs confirmation The mechanism of the vitamin action has been traced definitely for three vitamins of the B complex, namely for vitamins $B_1$ $B_2$ and nicotinic acid, which were found to take part in enzyme systems which are concerned with the processes of carboxylation and decarboxylation and by transportation of hydrogen with the oxidation reduction mechanism

## 3 Nomenclature

Hopkins[6] (1906–1912) called the nutritional elements which are required by the animal organism in addition to the carbohydrates fats proteins salts and water accessory factors In systematizing the knowledge of these accessory factors Funk[7] in 1912 proposed the generic term vitamine because these compounds are essential to life (the Latin term vita meaning life) and because he believed the anti beriberi compound to be an amine Funk[8] differentiated an anti beriberi vitamine an anti scurvy vitamine, an anti rickets vitamine etc *according to the diseases which* occurred during the respective nutritional deficiencies No statement could be made at that time as to whether these diseases are caused by a deficiency of just one compound or of a multitude of compounds Osborne and Mendel,[9] and McCollum and Davis [10] in 1915 distinguished two types of accessory factors by difference of their solubility and called them Fat Soluble A and Water Soluble B Fat soluble A was shown to cure a

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6 F G Hopkins *Analyst* 31 385 (1906) *J Physiol* 49 425 (1912)
7 C Funk *J State Med* 20 341 (1912)
8 C Funk *Die Vitamine* Wiesbaden 1914
9 T B Osborne and L B Mendel *J Biol Chem* 20 379 (1915)
10 E V McCollum and M Davis *Ibid* 23 181 (1915)

nutritional eye disease (the symptoms of which are keratomalacia) and to be necessary for growth of exp A deficiency of water soluble B produced beriberi terminology did not include a term for the anti scurvy mond[11] in 1920 proposed to combine the previously suggested te drop the terminal e of vitamine to the generic term vitamin and call the fat soluble growth and eye factor vitamin A, the water soluble anti beriberi compound vitamin B and the anti scurvy factor vitamin C This suggestion has generally been adopted by all workers in the field and the more recently discovered vitamins have been added to the list by using the letters of the alphabet consecutively Thus McCollum called the antirachitic compound vitamin D The anti sterility compound was called vitamin E etc The vitamins G, H and I were added later to the list of vitamins The blood coagulation vitamin was called vitamin K because the term Koagulation is spelled with 'K in the German language The postulated *l*actation vitamin is called vitamin I and the compound which prevents excessive *p*ermeability of cells is vitamin P

Considerable discussion arose from time to time as to whether or not the generic term vitamin should be maintained or changed to a term which would not indicate any relation to chemical or physiological properties of the compounds The term accessory factors of foods was discarded because it was felt that this term is too modest since the compounds in question are essential food factors and not merely accessory food constituents Other terms which have been suggested are Advitant and Exogenous Hormones Neither of these terms has however been generally accepted

The further development of the vitamin terminology has unfortunately resulted in an illogical nomenclature which can only be understood from its historical development What was originally called vitamin B proved to be a mixture of compounds which are referred to today as the vitamin B complex in accordance with the original nomenclature This complex consists of an unknown number of different vitamins which have been designated arbitrarily as vitamin $B_1$ $B_2$ etc The anti beriberi vitamin which was first discovered is called vitamin $B_1$ So far eight different vitamins have been given vitamin B subnumbers One of the difficulties which arose during the course of the vitamin research was that a deficient diet gave different symptoms in various animals although it was found later that some of these symptoms were caused by the absence of the same vitamin Thus vitamin $B_2$ has also been called vitamin G Originally vitamins B

J C Drummond Biochem J 14 660 (1920)

and $B_4$ were believed to be necessary only for the rat while vitamins $B_3$ and $B_5$ were believed to produce growth only in the pigeon

By the time that the vitamins were isolated as chemical compounds, they were given names which identified them according to the class of chemical compounds to which they belong Thus, vitamin $B_1$ is called 'thiamin," vitamin $B_2$ ' riboflavin,' vitamin $B_6$ pyridoxin,' etc While the research on the isolation of the vitamins $B_3$, $B_4$ and $B_5$ was progressing only slowly, other vitamins were discovered through experimental work with different organisms Thus nicotinic acid pantothenic acid and biotin which were known to be growth stimulants for yeast were also found to be vitamins for the animal organism It has subsequently been discovered that pantothenic acid is probably identical with vitamin $B_3$ and nicotinic acid with vitamin $B_5$ Biotin, glycine and arginine, on the other hand, appear to be present in what was originally called vitamin $B_4$

While the original vitamin B has been shown to consist of a number of *chemically and physiologically different compounds which were differentiated* by subnumbers, the fat soluble vitamins A, D E and K each have been found to occur naturally not as single compounds but as mixtures of compounds each of which exerts the same physiological action but differs from the others slightly in its chemical constitution Thus there exist two or three different vitamins A, at least six vitamins D, three vitamins E and two vitamins K They are called vitamins A D, E and K and are differentiated by subnumbers Thus, for example vitamin $A_1$ exerts essentially the same physiological effect as vitamin $A_2$ On the other hand in the water soluble class of vitamins each subnumber indicates a physiologically different vitamin with an entirely different mechanism of action as has been stated before

The vitamins have been classified as biocatalysts together with the *hormones and enzymes* Instead of the term biocatalysts ' the term ' ergins has been proposed by Ammon and Dirscherl [12] On the other hand Euler[13] advocated the term ergons for the vitamins, hormones and those coenzymes which contain as part of their structure vitamin or hormone molecules Such coenzymes have also been called vitazymes and ' hormozymes '

## 4 List of the Vitamins

Two classes of vitamins are differentiated today, namely, those which have been identified and those which have not been definitely identified

[12] R Ammon and W Dirscherl *Fermente Hormone Vitamine* Leipzig 1938

[13] H v Euler *Arkiv Kemi Mineral Geol.* A11 No 12 (1934) B11 No 45 (1934) B12 No 11 (1935)

By identification is meant the recognition of a vitamin as a chemical compound the physical properties of which have been established

THE IDENTIFIED VITAMINS

| Vitamin | Name | |
|---|---|---|
| The Group of Vitamins A | | |
| Vitamin A | Axerophthol | |
| Vitamin $A_2$ | | |
| Vitamin $A_3$ ? | | |
| Vitamin $B_1$ | Thiamin | Vitamin B Complex |
| Vitamin $B_2$ | Riboflavin | Vitamin B Complex |
| Vitamin $B_6$ | Pyridoxin | Vitamin B Complex |
| Nicotinic Acid (Vitamin $B_5$ ?) | | Vitamin B Complex |
| Pantothenic Acid (Vitamin $B_3$ ?) | | Vitamin B Complex |
| Inositol | | Vitamin B Complex |
| *p* Amino Benzoic Acid | | Vitamin B Complex |
| Vitamin C | Ascorbic acid | |
| The Group of Vitamins D | | |
| Vitamin $D_2$ | Viosterol calciferol | |
| Vitamin $D_3$ | | |
| Vitamin $D_4$ | | |
| Vitamin $D_5$ | | |
| Vitamin $D_6$ | | |
| The Group of Vitamins E | Tocopherols | |
| $\alpha$ Tocopherol | | |
| $\beta$ Tocopherol | | |
| $\gamma$ Tocopherol | | |
| Vitamin H | Biotin | |
| The Group of Vitamins K | | |
| Vitamin $K_1$ | | |
| Vitamin $K_2$ | | |
| Vitamin P | Citrin | |

THE NON IDENTIFIED VITAMINS

Vitamin $B_3$ (Pantothenic Acid ?)
Vitamin $B_4$ (Biotin Arginine Glycine ?)
Vitamin $B_5$ (Nicotinic Acid ?)
Vitamin $B_7$—Vitamin I
Vitamin $B_8$ (Adenylic Acid)
Vitamin B
Vitamin $B_p$ (Anti Perosis Vitamin)
Vitamin J

Group of Vitamins L (Lactation Vitamin)
Vitamin $L_1$
Vitamin $L_2$
Vitamin M
Factor T
Factor U
Folic Acid
Grass Juice Factor

A list of the vitagens is as follows

THE VITAGENS

| | |
|---|---|
| Essential fatty acids | Choline and related compounds and the essential transferable methyl group |
| Essential amino acids | Essential organic sulfur containing compounds |
| Essential carbohydrates | |

## 5 Occurrence

The natural distribution of the vitamins is discussed in this monograph separately in the vitamin chapters under the heading of occurrence Generally speaking vitamins occur in plant materials and are found in the animal organism only as a result of food intake or of the anabolic activity of micro organisms living in the intestinal tract The vitamin *content of various sources is of eminent* importance for the proper selection of food for man and animals and is often the determining factor in the commercial preparation of vitamin concentrates Nevertheless, quantitative data as to the vitamin content are given only occasionally in this monograph For practical purposes it is not possible to assign definite data to the vitamin content of foods because the vitamin content is only to a limited extent a function of the vitamin concentration in the fresh vegetable in the fresh fruit etc The amount of most vitamins contained in food after cooking or preservation procedures differs considerably from that of the untreated food In most cases the vitamin content decreases during cooking heating and washing procedures while in some instances the amount of available vitamins increases by liberation from protein material

*Among the many other factors* which influence the vitamin content of vegetable foods are the species the time of harvesting and the soil And the potency of animal foods depends somewhat upon the vitamin supply in the animal s diet

Extensive studies have been made to learn the vitamin content of practically all foods and foodstuffs treated and untreated The available information has been collected in a number of monographs [14] which are recommended as an excellent guide for the approximate vitamin potency of foodstuffs

[14] F P Daniel and H E Munsell *United States Department of Agriculture Misc Publ* No 27 (1937) L E Booher E R Hartzler and E M Hewston *United States Department of Agriculture Circ* No 638 (1942) M A B Fixsen and M H Roscoe *Nutrition Abstracts and Reviews* 9 7 (1940) A L Bacharach *Ibid* 10 4 (1941) H A Waisman and C A Elvehjem *The Vitamin Content of Meat* Burgess Publ Co Minneapolis 1941

## 6 Isolation

The first step in the recognition of a new vitamin is to produce a nutritional deficiency in an experimental animal The next step consists in the isolation of the compound which exerts vitamin activity The procedure of isolating the unknown vitamin is very tedious and requires in many cases a million fold concentration of the original material In the beginning of the history of vitamin research no effective methods were available for the isolation of compounds which occur in such small amounts and in mixtures with such enormous quantities of other substances New methods therefore, had to be devised and many old methods which were effective enough for the isolation of other compounds had to be modified and refined The most important new methods are the chromatographic adsorption technic and the high vacuum molecular distillation Both of these methods have been developed to such an extent that they are now used for the isolation of many vitamins in the pure form and have found commercial applications

Under the heading Isolation the methods used for the isolation of each vitamin are discussed separately in the respective chapters

## 7 Chemical Constitution

Once a vitamin has been isolated in the pure or essentially pure state efforts are made to elucidate the chemical constitution The methods for the analysis and determination of the structure are principally the same as those which have been used for the determination of the structure of many organic compounds of non vitamin character They consist essentially in the degradation of the unknown compound to smaller molecules until derivatives of known structure are obtained The structure of the original vitamin molecule is then reconstructed by skillful organic chemical reasoning

Since each of the vitamins has a structure which is entirely different *from that of any other vitamin it is necessary to present the work carried* out in determining the chemical constitution of the vitamins separately for each vitamin Therefore the results of the experimental work which led to the postulation of the structure of the compound are reported under the individual vitamins

## 8 Synthesis

The normal consequence of a postulation of the constitution of a vitamin is a challenge to synthesize the vitamin Actually all vitamins which

have been isolated have been synthesized with the exception of biotin (for which no structural formula has been suggested as yet due to the fact that sufficient amounts of the pure vitamin have not been available for chemical studies) A total synthesis is regarded as the final proof for the formula which has been postulated on the basis of degradation reactions The methods used for the synthesis of the various vitamins are presented in special chapters No total synthesis of any one of the vitamins D has been accomplished as yet, but some of the provitamins D have been obtained synthetically by hemi synthesis from various sterols

## 9 Industrial Methods of Preparation

An important feature in the development of vitamin research has been the availability of the vitamins to the clinician and practitioner for studies on experimental animals, for use in human therapy and finally for incorporation into foodstuffs for man and animals The first industrial methods which were used in the production of vitamin preparations for the public were extractions from natural sources Originally crude concentrates were offered to the trade but recently the methods of isolation have been refined to such a degree that it is possible to offer commercially practically all the vitamins in essentially pure state Besides the isolation of the natural compound synthetic methods of preparing the vitamins have been studied and developed to such an extent that it is now more economical to produce most vitamins on a commercial scale by synthesis than by extraction from natural sources

The methods used in industry for the manufacture of vitamin preparations are known in general but the details are usually kept secret Under the heading of industrial methods of preparing vitamins the procedures used commercially are briefly reviewed for each vitamin The result of industrial research and development work is reflected in patents which have been taken out to protect the manufacturers and the public, both of whom are interested in obtaining the best possible products and preventing unskilled and dishonest manufacturers from exploiting the market [15] A list of the issued patents from the main industrialized countries United States of America, Great Britain, Germany and France will be found at the end of this monograph arranged according to subject In isolated cases, patents issued in Switzerland Holland Belgium, Canada Japan and Russia have also been incorporated

[15] A C Connolly *Science* 86 383 (1937)

## 10 Biogenesis

The methods used in the plant organism for synthesizing vitamins which are complex compounds, are very interesting from a physiological stand point While up to the present time these methods are not known for all the vitamins and only a few theoretical approaches have been made, it is worth noting that the building principles for the synthesis of vitamins are the same as those used by the plant organism for the synthesis of many structural units Thus we find there is a vitamin represented among practically all types of compounds synthesized in plants Further research will elucidate the reaction mechanism used by the plant organism for the synthesis of the structural building units and hence for vitamins in particular Ultimately, the physiologist wishes to determine why vitamins are synthesized, why they are produced only in such minute quantities and how the mechanism for synthesizing these particular compounds differs from that for other chemically very closely related substances which have no vitamin action

## 11 Specificity

One of the most fascinating studies in biochemical research is the determination of the specificity of the vitamins Generally speaking, two types of specificity must be differentiated namely, compound specificity and species specificity By compound specificity is meant the qualitative and quantitative differences in physiological behavior of various compounds on the same animal organism Species specificity is defined as the difference in physiological response of one or more compounds on different species of animals The various forms of vitamin D may serve as an illustration for both the compound and species specificity Vitamin $D_2$ (activated ergosterol) and vitamin $D_3$ (activated 7 dehydro cholesterol) have qualitatively and quantitatively the same antirachitic activity when tested on rats Vitamin $D_4$ (activated 22 dihydro ergosterol) has qualitatively the same antirachitic efficacy but quantitatively the effect is only half as great as that of the vitamins $D_2$ and $D_3$ on the molecular weight basis This effect demonstrates compound specificity When the same three vitamins D are tested on chicks under standardized conditions on the basis of Rat Units and compared with cod liver oil it is observed that vitamin $D_3$ is as active as the vitamin D in cod liver oil and this activity is arbitrarily designated as 100% activity On the other hand when vitamin $D_2$ is tested under the same conditions an activity of only a few per

cent is obtained Vitamin $D_4$ exerts an activity of about 20% under the same conditions This effect demonstrates species specificity

One of the objects of studying the specificity of vitamins is to determine whether or not the vitamin activity is due to the structure of the entire molecule or to a special group This type of research has proved to be of extreme importance in the study of many pharmaceutical compounds A well known example in the field of chemotherapeutical compounds is the research which followed the discovery that "prontosil" counteracts strep tococci[16] Investigations revealed that the bactericidal action is due solely to the sulfanilamide portion of the molecule[17] Research of this type in the vitamin field brought to light that the activity exerted by cho line is due to the available methyl groups of the compound and resulted in the recognition of the available methyl group as a vitamin (or vitagen) factor Specificity studies on the group of vitamins K showed that while the naturally occurring vitamins $K_1$ and K are 2 methyl 1,4 naphtho quinone derivatives with long side chains, the vitamin activity is due only to the 2 methyl 1 4 naphthoquinone portion of the molecule

Specificity studies in other fields have revealed that it is possible to alter the properties of the active compound so as to make them more suitable for therapeutic use One of the best known examples in the hormone field is the use of methyl testosterone[18] which does not occur naturally but which is effective when given by mouth[19] whereas the naturally occurring testosterone is active only by injection Alterations of chemical or physi cal properties without alteration of physiological action in the vitamin field are of importance, for example in order to make fat soluble vitamins water soluble or water soluble vitamins fat soluble The most important practical problem of this type concerns vitamin K K avitaminosis occurs in patients with obstructive jaundice due to the fact that the bile does not reach the intestinal tract and thus prevents the vitamin from being ab sorbed On the other hand water solubilized forms of vitamin K do not require the presence of bile for absorption from the intestinal tract and are thus active as such when taken orally

From the vast amount of work which has been done to elucidate the com pound specificity of the vitamins it can be concluded that the physiological action of vitamins is very specifically a function of the entire molecule In the class of water soluble vitamins it is observed that lower or higher

[16] C Domagk *Deut Med Wochschr* 61 250 (1935)
[17] J Tréfouel J Tréfouel F Nitti and D Bovet *Compt rend soc biol* 120 756 (1935)
[18] L Ruzicka M W Goldberg and H R Rosenberg *Helv Chim Acta* 18 1487 (1935)
[19] K Miescher and W Tschopp *Schweiz Med Wochschr* 68 1258 (1938) I G l o s *Brit Med J* 1939 July 1 No 409 11

homologs have either much lower activity than the vitamin proper or have no vitamin activity whatsoever This is not the case in the class of fat soluble vitamins Actually they occur naturally as a mixture of various homologs Geometrical isomers of the vitamins are usually considerably less active than the vitamin itself Thus activated epi 7 dehydro choles terol is only about one tenth as active as activated 7 dehydro cholesterol Another example of the high specificity of the vitamins is the specific ef fect of *d* ribose in the vitamin $B_2$ molecule This sugar compound cannot be replaced by any other sugar without essential loss of activity

Under the heading Specificity the results obtained from the experi mental studies and the conclusions reached will be presented separately for each vitamin

## 12 Determination

One of the most fruitful developments in the biological field has been the study of methods for measuring the potency of vitamin preparations There are principally a number of different procedures for the determina tion of vitamins These comprise physical, chemical biochemical and biological methods Generally speaking any method has to be investi gated thoroughly in order to determine the specificity afforded by the test the types of compounds which inhibit an evaluation of the procedure and finally the sensitivity of the procedure

Among the physical methods for the determination of vitamins spectro scopic studies are outstanding These include determinations of the ab sorption spectrum in the ultraviolet or visible light region The fluores cence spectrum is used successfully in specific cases These tests are based on the presence of a specific chemical group in the vitamin molecule Therefore this procedure cannot be specific for any vitamin since the same physical phenomenon is given by any other chemical compound with the same characteristic group Nevertheless the physical methods are quite accurate when appropriately used and are in general more rapid than other methods

The basis for all determinations of absorption spectra is Beer s law

$$I = I_0 10^{-\epsilon c d}$$

In this formula $I_0$ stands for the intensity of the incident beam $I$ for the intensity of the transmitted beam $c$ for the concentration (expressed in mols per liter) $d$ for the depth of the solution (in cm ) and $\epsilon$ for the ex tinction coefficient There is however no uniformity in the use of the units

As long as a compound has not been isolated in the pure form merely transmissions of solutions of known concentration are plotted and expressed as $\log I_0/I = \epsilon c d$ The adoption of a solution of 1 cm depth standardized to contain 1% of the substance and expressed as *extinction* $E^{1\%}_{1cm} = \log I_0/I$ is a frequently used refinement in the study of absorption characteristics The *absorption constant* $K$ has also frequently been used and is defined in the formula

$$I = I_0 e^{-Kcd}$$

($e$ is the basis of the natural logarithm) When the molecular weight of a compound is known, the extinction of the absorption per mol is measured and expressed according to Beer's law either as

$$\epsilon = \frac{1}{cd} \quad \log \frac{I_0}{I}$$

or

$$\kappa = \frac{2\,3}{cd} \quad \log \frac{I_0}{I} \left[\frac{10^3\,\mathrm{cm}^2}{g\ \mathrm{mol}}\right]$$

The chemical methods for the determination of vitamins are based upon certain chemical reactions which the vitamin molecule undergoes with specific reagents Usually those types of reactions are used for the determination of vitamins which develop a color which can be measured quantitatively Such methods have been worked out for practically every vitamin and can be carried out relatively quickly However they have the disadvantage that the reactions are not specific for the vitamin since the same or similar color reactions are also given by a series of chemically similar and different compounds

Biochemical methods for the determination of vitamins are defined as procedures in which the vitamin is determined by the part it takes in certain biochemical reactions An illustration is the action of vitamin $B_1$ in a specific enzyme system which decarboxylates pyruvic acid These procedures are very specific for the particular vitamin which is determined but require special skill in handling enzyme systems and are usually quite tedious They are used in special cases with considerable advantage over any of the other procedures

All physical chemical and to a certain extent also the biochemical methods for the determination of vitamins are valid only if their results can be related to actual biological values Thus, the biological methods which

have generally been used as the primary tool in the discovery of the vitamins, must still be resorted to today as the final criteria for qualitative and quantitative vitamin assays and for standardization and determination of the accuracy of other methods. Biological methods are very time consuming and costly. The primary requirement is to feed a group of comparable animals which are used for the determination of vitamins a special diet which is deficient only in the vitamin to be tested and is otherwise well balanced in regard to the other vitamins and the energy and building unit bearing and protective food constituents. Multiple deficiencies, that is, the concurrence of avitaminoses caused by lack of more than one vitamin should generally be avoided in biological vitamin assays. The actual determination can be carried out either on a curative or on a prophylactic basis. The curative methods are usually preferred since it is possible to use for the actual test only those animals which have been inflicted successfully with the deficiency disease. It is often necessary to carry out simultaneously with the vitamin determination check experiments with animals which have not obtained the vitamin supplement. The highest possible degree of accuracy is achieved by comparing in parallel experiments the activity of the unknown material with the activity of a standard preparation. The results of biological assays are evaluated statistically. Mathematicians and biologists have given much thought as to the method of carrying out this statistical evaluation most efficiently in order to achieve a high degree of accuracy with a minimum of experimental animals.

A special type of biological method for the determination of vitamins is the so called microbiological method in which microorganisms are used as test objects. Thus practically all members of the vitamin B complex can be determined by the growth effect which they exert on microorganisms, such as yeast and different strains of bacteria. The amounts of vitamin required for such microbiological assays are considerably smaller than are needed in experiments with higher animals. The microbiological tests are relatively inexpensive and can be carried out in a relatively short period of time. Especially sensitive are such microbiological methods which are based not on the growth of the organism but on the action of the organism in special culture media. Thus those bacteria are especially useful which produce acids, for example lactic acid. The actual determination of the vitamin effect involves in such cases a titration of the amount of acid formed. This type of biological vitamin assay has proved to be of high accuracy in the determination of pantothenic acid, nicotinic acid and biotin.

The special methods which have been used and recommended for the determination of each vitamin are presented and discussed separately under each vitamin

### 13 Standards

Standards of vitamins are necessary for the determination of these protective food constituents and for proper dosage Vitamins originally were defined in terms of biological units However, as soon as a vitamin is obtained in crystallized form and is easily accessible in that form, the standard is expressed on the weight basis of the crystallized material

The term biological unit' is a generic term for specific animal units such as Rat Unit, Chick Unit, Mouse Unit, etc The biological units of non identified vitamins are of relatively uncertain definition They represent the reciprocal of a dose which had a certain effect on a certain animal at a certain time Thus a biological unit refers to the action of an animal while a standard based on the weight unit refers to the amount of the vitamin

During the last two decades, national and international standards have been set up for many vitamins, especially for the non identified vitamins By the time a vitamin is available as a stable crystallized compound a new standard is set up which defines the unit in terms of actual weight of the vitamin Usually that amount of crystallized vitamin is defined as one unit which corresponds on a comparative basis to the effect of the original biological unit

The standards as adopted or as recommended for adoption are presented for each vitamin separately in the corresponding chapters

## PHYSIOLOGY

The physiology of vitamins comprises a study of the vitamin action in plants and animals Since by definition specific organic compounds which are indispensable to the animal organism are called vitamins and since the definition contains no statement concerning plants, it is obvious that the physiological action of the vitamin compounds in the animal organism is considered to be of primary importance Nevertheless the physiological action of these compounds in plants where most vitamins are synthesized, must be incorporated in a complete study of the vitamins Animal physiology is subdivided with respect to the action of the vitamins in the organism into (a) the metabolism of the vitamins (b) the physiological action of the vitamins (c) the mechanism of the vitamin action and (d) the relation of the vitamins to each other to hormones and minerals

The sections on the physiology of the vitamins are presented in a somewhat different manner from that of the sections on the chemistry of the vitamins due to the different character of the subject The knowledge of both the chemistry and the physiology is based on experimental work There is no, or practically no misunderstanding possible in the conclusions drawn from intelligently planned chemical experiments This is due to the fact that chemical experiments deal generally with systems the constituents and the reactions of which are known and contain as the only unknown the vitamin The situation is entirely different in experiments designed to elucidate the physiology of the vitamins or the mechanism of the vitamin action Here the vitamin acts in a very complicated and largely unknown system namely, the living organism As a result all experimental data obtained from studies on the living organism must be qualified as to the exact conditions used in the experiments The multiplicity of possible interpretations of these results then causes difficulties The same physiological experiment carried out under only slightly different conditions may give entirely different results The interpretations of these results are then bound to differ markedly By the time the knowledge of the chemistry and the physiology of the vitamins and the living organism expands it is evident that conclusions drawn from earlier experiments must often be modified due to the fact that all statements regarding physiological reactions are based on certain assumptions and hypotheses regarding the equivalency of principal physiological reactions under various reaction conditions

The result of all these factors is that a tremendous amount of work is necessary to establish the true physiological action of the vitamins In a few cases this has already been acomplished In most cases a final understanding has not been reached A review of these latter cases is extremely difficult since from the mass of data and interpretations available only a limited amount can be presented in this monograph The discussion of the experimental data is usually followed by interpretations which are mostly subjective in nature Efforts have been made to present the material in the form of a well rounded picture but it appears possible that just this attempt has led to the presentation of conclusions which ultimately may prove to be erroneous A few examples of somewhat questionable views may be cited The existence of a combined form of ascorbic acid ascorbigen is accepted as proved in at least a few instances although many references could be cited to disprove the theory of the existence of ascorbigen The view is taken that vitamin D is less efficacious for human beings than is vitamin $D_3$ although statements disproving this thesis can be found

in the literature In most cases in which a distinct difference of opinion exists between various groups of workers both sides are cited

## 14 Physiology of Plants and Microorganisms

Most vitamins are synthesized in plants There is apparently a special mechanism for the synthesis of each of the vitamin compounds Very little is known about the physiological behavior of these compounds in plants, but it has been shown in a few isolated cases that the vitamins are essential growth promoting substances for the plant organism It is assumed that the vitamins act in the plant organism principally in the same manner in which they act in the animal organism

The information available concerning the physiological action of the vitamins in microorganisms is very meager The most interesting discovery in this field is the observation that while some microorganisms are able to provide their own needs like the plants there are others which rely upon an outside supply of the vitamins Curiously enough there are also quite a number of microorganisms which do not have the power of synthesizing the vitamins completely, but which are able to synthesize part of the vitamin molecule and are able to build up the entire molecule when the *missing component is supplied*

## 15 Animal Physiology

### (a) *Metabolism of the Vitamins*

Vitamins are by definition compounds which cannot be synthesized in the organism of man or animals With one single exception (vitamin D) the vitamins are always supplied to the human and animal organism by oral administration as normal food constituents In the properly functioning organism they are readily absorbed in the intestinal tract The water soluble vitamins are absorbed as such at least when they are supplied in the free form In combined form they have to be hydrolyzed to the free vitamin before absorption can take place The fat soluble vitamins on the other hand require the presence of bile for proper absorption Following the absorption the vitamins are carried through the organism by means of the blood stream and are carried to the tissues and organs which need them

The animal organism does not have special storage organs for the deposition of vitamins A certain level of vitamins is usually maintained in tissues and body fluids and some special organs contain increased amounts The reason for the presence of these increased amounts is not obvious,

but is believed to be a necessity for the proper functioning of that particular organ This conclusion has been reached in recognition of the fact that during times of low vitamin intake these organs are not depleted of their vitamin content, although the actual amount is usually somewhat reduced Simultaneously, symptoms of vitamin deficiencies occur long before the amount present in these special organs is exhausted Thus, the amount of vitamins present in such special organs cannot be considered as a storage of the vitamin for distribution over the entire organism

The physiological reason for maintaining a certain normal vitamin concentration in the organism is apparently a certain safety factor for times of low vitamin intake To a limited extent the body appears to contain a self regulatory mechanism for the utilization of the vitamins This is apparent from the fact that while the healthy organism on a normal diet excretes certain amounts of the vitamins they are not excreted or are excreted only to a limited extent at times of low vitamin intake even when abundant quantities are present in the organism

Administration of excessive amounts of the vitamins usually causes their excretion within a relatively short time The water soluble vitamins are excreted mainly through the urine The fat soluble vitamins are excreted through the feces A certain amount is destroyed in the organism It appears that once a vitamin molecule is destroyed it is completely burned since it has not been possible so far to find any degradation products in the excretions

In accordance with the life maintaining function of the vitamins it is obvious that these protective compounds are secreted in the nutritional materials necessary for proper growth and development of the offspring Thus small but definite amounts of all vitamins are found in milk and eggs

## (b) *Physiological Action of the Vitamins*

The principal physiological action of vitamins has become a part of the vitamin definition They do not furnish energy and are not utilized as building units for the structure of the organism but are essential for the transformation of energy and for the regulation of the metabolism of structural units

Vitamins are necessary for the animal organism as a whole not for the individual cells although the reactions of the vitamins are carried out predominantly in cells Cells deprived of vitamins can survive and multiply while the entire organism cannot [20]

[20] S B Wolbach *Science* 86 569 (1937)

The primary physiological action of most vitamins is scarcely known The observations made in experimental studies are in many cases secondary symptoms or secondary reactions, and it is very difficult to decide in many cases whether the observed actions of the vitamins are of primary or of secondary nature Whatever they may be it seems that, at least on the basis of the present day information, a certain difference exists between the physiological action of the water soluble and fat soluble vitamins The fat soluble vitamins and also water soluble vitamin C apparently maintain the 'regulation of the metabolism of structural units ' From the experimental data available it may be concluded that vitamin A is concerned with the building of the cell nucleus and vitamin E with the maturation and differentiation of cells Vitamin C enables the cells to produce supporting tissues and to maintain intercellular substances Vitamin D plays an essential role in the process of bone calcification, which process is a deposition of calcium phosphate in bone matrix Vitamin K maintains the level of certain structural (or at least potentially structural) building units of blood which initiate the process of blood coagulation under proper conditions In contradistinction from the action of these vitamins, the water soluble vitamins of the B complex" are concerned mainly 'with the mechanism of the transformation of energy ' Thus the vitamins $B_1$, $B_2$ and nicotinic acid have been shown to take part in certain reactions which regulate the carbohydrate metabolism Vitamin $B_6$ is apparently involved in the amino acid metabolism

The observations and conclusions reached from experimental data concerning the physiological action of the vitamins in the animal organism are presented separately for each vitamin in the corresponding chapters

### (c) *Mechanism of the Vitamin Action*

Ultimately the mechanism of the action of each vitamin will be understood So far the reaction mechanism of only a few vitamins is known The primary requisite for studies of the mechanism of the vitamin action is a proper understanding of the vitamin action as such As has been pointed out in the previous paragraph, the physiological action of the vitamins is as a whole not properly understood As a result the mechanism is unknown In the few cases in which the primary physiological action of a vitamin has been elucidated efforts have been made to determine the mechanism of the vitamin action Thus, in the cases of the vitamins $B_1$ B and nicotinic acid of the vitamin B complex the relation of the vitamin to the carbohydrate metabolism has been the subject of many experimen

tal studies and theoretical considerations These have culminated in the recognition of the fact that these three vitamins are part of enzyme systems which can be essentially separated from the animal organism and can thus be subjected to experimental studies under defined conditions in contradistinction to the reactions in the organism where the conditions are not defined at least not from an experimental point of view As a result of this the mechanism of the action of these three vitamins is at least partly understood

The mechanism of the action of the other vitamins is not known but various theories have been presented which attempt to explain the experimentally observed phenomena

*(d) Relation of the Vitamins to Each Other to Hormones and Minerals*

An integral part of the physiological action of the vitamins is their influence on the secretion of hormones and on the metabolism of inorganic compounds However no physiological relation exists between any one vitamin and another

Shortly after the recognition of the existence of vitamins a theory of synergism and antagonism of the vitamins was postulated According to this theory the elimination of one vitamin from the food causes a change of the action of the remaining vitamins Certain experimental facts have been cited to prove this conception but a logical consideration of the vitamin concept disproves the existence of this theory If it were true that the elimination of one vitamin changes the action of the remaining vitamins it would be impossible to observe specific vitamin deficiencies It must be remembered that the vitamin intake of the average human being changes considerably from day to day It is quite certain that the amount of vitamins administered every day changes markedly Thus, on one day a considerable amount of one vitamin may be consumed while the diet of the next day may not contain any of this vitamin If the theory of synergism and antagonism of vitamins were correct each vitamin would thus exert a different type of reaction practically every day The knowledge of the vitamin action and of the mechanism of the vitamin activity of certain members of the vitamin B complex makes it appear improbable if not impossible that one vitamin could replace another in these reactions

Nevertheless the theory of antagonism and synergism of vitamins has been generally accepted although the experiments on which such a theory is based are not conclusive and have not been carried out under convincingly well chosen conditions Recent carefully controlled investiga

tions[21] have indicated that the vitamins A B₁ B complex and D do not act according to the postulated theory at least as long as administered in amounts which correspond to the optimal requirement of these vitamins It has thus been shown experimentally that a definite relation of one vitamin to another does not exist

It has been found that certain vitamins, especially vitamin C, but also vitamin A and vitamin B are able to exert in addition to their vitamin action also other therapeutic effects Thus, vitamin C has been found to counteract intoxications caused by many organic and inorganic compounds toxins etc This action is not specific for vitamin C but is a function of a particular group in the molecule Other compounds with similar chemical groups are able to exert the same or similar effects This detoxifying action of vitamin C has also been observed in certain experimental animals when excess toxic doses of fish liver oils have been administered The *beneficial effect of vitamin C has in this* case been interpreted as indicating an antagonism of vitamin C to the vitamins present in fish liver oils Actually however vitamin C acts as a detoxifying agent for the harmful components of the liver oil regardless of whether or not they are vitamins

Although the author of this monograph is of the definite opinion that the theory of synergism and antagonism of vitamins is incorrect and that such *actions do not* exist *theories concerning these phenomena and experi*mental data obtained in studies of these effects are presented in the corresponding section of each vitamin This presentation may assist in the ultimate recognition of the non existence of functional relations of one vitamin to another

In contradistinction to the relations of vitamins to each other there is the possibility that vitamins may influence the secretion or action of hormones Since the vitamins maintain animal life they may as part of their action influence secretions within the organism Thus a relation of vitamins to hormones is not illogical although not necessary *a priori* Thus it can be generally observed that in animals which have been deprived of vitamins for a long period of time not only the entire organism suffers but that specifically the glands lose vitality and as a result of this the effective secretion is reduced This has been especially obvious, for example in the case of the germinal glands In addition to this general effect of vitamins on the secretion of hormones there has also been postulated a theory of synergism and antagonism of vitamins to hormones In a few isolated cases indications for such relations have been observed Thus

[21] P E Simola *Ber ges Physiol exptl Pharmak 1* 83 312 (1936) A Scheunert *Naturwissenschaften* 28 297 (1940)

there is what has been interpreted as a synergism between vitamin C and the hormones of the adrenal medulla and cortex An antagonism between vitamin C and the thyroid hormone has also been postulated It is extremely difficult to prove or disprove experimentally the existence of relations of this type between vitamins and hormones For a long time such a relation had been assumed to exist between the hormone of the thyroid gland and vitamin D but more thorough studies have indicated that such a relation actually does not exist

It has already been stated under Physiological Action of the Vitamins (page 27) that they are concerned with the regulation of the metabolism of the structural units Thus a relation of the vitamins to those inorganics which act as structural building units is obvious In addition a relation of the vitamins to the functional inorganic building units of the organism exists Such a relation is however indirect in the sense that certain inorganics act vitamin like in addition to the vitamins and in co operation with them An antagonism or synergism is not known As an example of such a cooperative action may be cited the essential action of manganese in enzyme systems which contain vitamin $B_1$ and the action of copper in ascorbic oxidase

## PATHOLOGY

The study of the pathological effects caused by vitamins comprises a study of the effects caused by an administration of insufficient amounts of the vitamins and of excess doses The symptoms caused by the former conditions are called avitaminoses and hypovitaminoses whereas the symptoms caused by the latter conditions are called hypervitaminoses

### 16 Avitaminosis and Hypovitaminosis

During times of vitamin deficiencies two types of clinical symptoms can be observed namely specific deficiency syndromes and general non specific effects The latter comprise what is generally known as ill health and constitute minor aches and a feeling of discomfort In experimental animals a premature aging also is observed Such conditions are usually not specific for a particular vitamin but may be caused by a deficiency of practically any vitamin The specific deficiency syndromes are classified according to the severity of the cases into hypovitaminosis and avitaminosis The state of hypovitaminosis is characterized by a partial vitamin deficiency, that is a state at which a vitamin is administered in suboptimal amounts Under these circumstances the general health of the organism

decreases and specific deficiency syndromes set in These are usually not very severe and can be cured easily by the administration of the proper vitamin A total deficiency of a vitamin causes the appearance of specific clinical symptoms in a much more severe state and is called avitaminosis These diseases can usually be cured at an early stage Organisms which have suffered from avitaminoses for a prolonged period of time cannot always be repaired completely The clinical symptom which remains after treatment with vitamins is called paravitaminosis

The special symptoms of hypovitaminosis and avitaminosis in human beings and in experimental animals are described separately for each vitamin in the corresponding chapters

### (a) *Clinical Test Methods*

While it is usually easy to diagnose an avitaminosis, the recognition of a hypovitaminosis is often difficult due to the fact that the symptoms are not very specific Methods of determining the state of hypovitaminosis are, therefore, of considerable importance Hand in hand with this problem goes the problem of determining the average or individual requirement for a specific vitamin in order to insure the success of a prescribed vitamin therapy Tests have therefore been developed and are being perfected in which the concentrations of vitamins in the organism can be determined quantitatively In general, such tests deal with the determination of the vitamin in blood where a certain level usually is maintained and in the urine or feces provided the vitamin is regularly excreted Special saturation tests are being developed for many vitamins in which either the amount of vitamin necessary or the time consumed is measured until a certain minimum concentration of the vitamin occurs in blood or in the excretions In addition to these general methods a number of specific tests have been developed for special vitamins which are based on particular functions of such vitamins such as for example the dark adaptation test for the determination of the presence of vitamin A and the capillary resistance test for the determination of the presence of vitamin C (or vitamin P)

## 17 *Hypervitaminosis*

Hypervitaminosis is according to the definition of the term the clinical syndrome which occurs when toxic amounts of a vitamin are administered

[33] G Mouriquand *Bull acad méd* 119 (3) 10 678 (1938)

A study of the hypervitaminoses constitutes therefore a study of the pharmacological effects of the vitamins All vitamins in the pure state are essentially non toxic even at doses which are many times as great as those of the daily food intake The vitamins also exert no or practically no specific pharmacological effects other than the specific vitamin action

In experimental studies designed to determine the toxic level of vitamins, it has been found that amounts which are as high as a thousand or million times that of the daily requirement may cause the occurrence of specific syndromes of hypervitaminosis Specificity studies of the vitamins are especially interesting from the toxicological standpoint since it has been observed that compounds which differ chemically from the vitamins only slightly may be considerably more toxic than the vitamin itself The pharmacological study of chemical compounds usually comprises also a determination of the lethal dose The lethal dose of most vitamins is unknown due to the fact that the vitamins are essentially non toxic

## 18 Requirements

The requirements of vitamins by man and various species of animals are most important from a practical point of view The determination of the actual amount needed proved to be a very difficult task due to the fact that the vitamin requirements vary with environment and other factors Efforts are being made to correlate the necessary amount to either body weight or food intake according to the function of the vitamin

Research is being directed to and national and international organizations are concerned with the problem of setting up standards for the requirements of the vitamins The latest and most outstanding efforts of this type have precipitated the Recommended Dietary Allowances as formulated by the Food and Nutrition Board of the National Research Council The information published by this organization is reprinted on page 613 of this monograph These allowances fix desirable levels and are considered to be relatively liberal They constitute norms fixed by a body of distinguished American students of nutrition available to the rest of the world for discussion and study They furnish a guide for consideration and help to focus the aims of further experiment in the light of which further revision of the standards is to be anticipated

The amount of vitamins which is recommended is expressed as the optimum quantity under average conditions There was a tendency at the beginning of vitamin research to recommend as a daily dose the mini

R A Williams J Franklin Inst 237 91 (1944)

mum amount of vitamin which is necessary to prevent the occurrence of clinical symptoms of a deficiency disease It was observed in the following years that the minimum amount as defined previously was not always the optimum amount which should not only prevent the occurrence of specific clinical symptoms of an avitaminosis but should also provide for certain safety factors Generally speaking the vitamin requirements vary with age environment and individual utilization of nutrients Babies generally, need increased amounts that is the vitamin requirements of babies cannot be correlated to body weight or food intake on the same basis as expressed for adults When children grow older they still need increased amounts of vitamins but somewhat less than adults There are some indications that aged people need increased amounts of vitamins, which seems to be due to the fact that the organism of older people utilizes food less well than adults The influence of environment upon the vitamin requirements is especially evident for those vitamins, the average requirement for which is correlated to food intake Thus, it has been found that approximately twice as much of vitamins $B_1$, $B_6$ and pantothenic acid are required by rats at 91° F than at 65° F [23]

The health and general well being of a nation is dependent to a considerable extent upon the proper vitamin consumption of its people Extensive investigations have shown that the normal food of the average man in most countries is deficient in many vitamins It has therefore been necessary to take definite steps for providing an adequate vitamin supply to everyone This can be achieved by supplying with the necessary vitamins such foods as are generally used Thus the Food and Nutrition Board of the National Research Council considers it good practice to fortify margarine with vitamin A and milk with vitamin D It is also recommended and in some countries ordered to reconstitute white flour to the original content of the vitamin B complex as found for example in whole wheat grain In the United States of America enriched flour must contain per pound 2 0–2 5 mg of vitamin $B_1$, 1 2–1 5 mg of vitamin $B_2$ and 16–20 mg of nicotinic acid, and may contain 250–1000 U S Pharmacopoeia Units of vitamin D [4]

[23] C A Mills *Proc Am Physiol Soc* 1941 *P* 202
[4] *Federal Register The National Archives of the United States* July 3 1943

# THE GROUP OF
# VITAMINS A

# THE GROUP OF VITAMINS A

## 1 Chronology

1831 WACKENRODER isolated carotene from carrots
1904 Xerophthalmia was observed as an epidemic disease in Japan
1906 The empirical formula of carotene was established by WILLSTATTER
HOPKINS and STEPP (1909) discovered the indispensability of certain fat soluble substances for the growth of mice and rats
1913–1915 McCOLLUM and DAVIS and OSBORNE and MENDEL ascertained in differentiation from other compounds the presence of a growth factor fat soluble A in cod liver oil and in butter
1919 STEENBOCK discovered the vitamin A activity of carotenoids (His results could not be verified until 1929 when H v EULER showed that vitamin D must be added to the diet of animals )
1920 STEENBOCK recognized that vitamin A is found in the unsaponifiable parts of fish oils
1928–1930 ZECHMEISTER KARRER and KUHN established the constitution of carotene
1930 MOORE disclosed the conversion of provitamins A to vitamin A in the animal organism and storage of vitamin A in the liver after large doses of carotene
1931 KARRER obtained a very highly concentrated vitamin A preparation and determined the structure of the vitamin
1933 KARRER synthesized perhydro vitamin A
1937 KUHN and MORRIS announced a synthesis of vitamin A HOLMES and CORBET obtained vitamin A for the first time in crystalline form In this year the existence of a second form of this vitamin called vitamin $A_2$ was recognized by LEDERER and EDISBURY and their respective co-workers

## 2 The Group of Vitamins A

The physiological effect of vitamin A is brought about in man and in animals by a number of different naturally occurring and synthetic compounds Whereas all these substances appear to react physiologically alike they differ from each other chemically The compounds found in plants with vitamin A activity for animals, belong to the class of carotenoids with 40 carbon atoms They are apparently not used as such by the organism (at least not as vitamin A), but are converted into other substances to produce vitamin A activity These new compounds are stored to a certain extent in the animal organism in special storage organs but

have never been found in plants Chemically, they are degradation products of the carotenoids The most familiar of these is the one known as vitamin A which possesses the empirical formula $C_{20}H_{30}O$ All degradation products of carotenoids which occur in the animal body and react physiologically alike are called "vitamins A." The carotenes the precursors of the vitamin A, are called "provitamins A" *It should be pointed* out that the term provitamin is applied properly only to the specific organism for which the physiological activity has been ascertained Rats are usually used for provitamin A studies and *it is assumed, but not proved*, that compounds of provitamin activity for rats also exhibit provitamin A activity for man Nine different naturally occurring provitamins A and two vitamins A are known However, there are many reasons for believing that more provitamins and especially, more vitamins A occur in nature

The true physiological action and the mechanism of the vitamin A activity are essentially unknown A few approaches have been made, however, toward solving this problem Furthermore, a number of different diseases of man and animals are known, which are caused by a vitamin A deficiency and which can be cured by the administration of the vitamin

## PROVITAMINS A

### 3 Occurrence

The provitamins A occur in plants together with chlorophyll They are generally absent from the animal organism, since they are not stored as such but are converted into vitamins A A few exceptions to this general rule must, however, be noted Traces of provitamins A have been found in fat deposits of animals A special type of this class of compounds plays an important role in the visual purple of the eye and will be discussed later (see page 89) Milk and butter contain small amounts of carotenes Egg yolk, however contains only traces Almost pure $\beta$ carotene (and some $\alpha$ carotene) has been found also in the *corpus luteum*[1] and the *corpus rubrum* of cows,[2] in the human placenta,[2] the testes of bulls[3] and the adrenal gland of practically all mammals[4] Carotenoids, the provitamin

[1] R Kuhn and E Lederer *Z physiol Chem* 200 246 (1931) P Karrer and W Schlientz *Helv Chim Acta* 17 8 (1934)

[2] R Kuhn and H Brockmann *Z physiol Chem* 206 64 (193 )

[3] R Netter *Bull soc chim biol* 14 1555 (1932)

[4] A A H van den Bergh P Muller and J Broekmeyer *Biochem Z* 108 279 (1920) C L Connor *J Biol Chem* 77 619 (1928) *Am J Path* 4 293 (1928) H v Euler and E Virgin *Biochem Z* 245 252 (1932) H v Euler U Gard and H Hellström *Svensk Kem Tid* 44 191 (193?) I Zechmeister and P Tuzson *Z physiol Chem* 231 259 (1935) O Bailly and R Netter *Compt rend* 193 961 (1931)

A character of which has not definitely been established occur also in the yellow bone marrow The sex glands of sea urchins contain a provitamin A, echinenone which has so far not been found in plant material

The most important sources of vitamin A for man and animals are the provitamins present in all green or yellow parts of vegetables Carrots apricots [5] lettuce cabbage and spinach are especially rich in carotenes Tomatoes and bananas contain a lesser amount Yellow corn contains a considerable quantity of a special provitamin A cryptoxanthene [6] predominantly in the form of its ester The presence of carotenes in certain vegetable oils especially red palm oil [7] is technically important The provitamins myxoxanthin and aphanin occur in algae especially blue green algae

The total amount of carotenes in vegetables is rather small Fresh carrots contain approximately 0 01% carotenes and the best source of palm oil 0 15–0 20% [8]

The provitamins A occur in nature only to a limited extent in the free form They are mainly bound in symplex form to proteins as has been demonstrated for carotene both in plant (carrots[9]) and in animal (serum[10]) materials

## 4 Properties

There are nine different naturally occurring compounds known as provitamins A namely $\alpha$ $\beta$ and $\gamma$ carotene, cryptoxanthene, echinenone myxoxanthin, leprotene aphanin and aphanicin It is quite possible that other provitamins A occur in nature which have not been discovered as yet All these substances crystallize in deep red prisms of high melting points (above 160°) They exhibit typical absorption spectra (listed below) the positions of the maxima of which differ with the solvent used The theory has been advanced that this shift is due to a change of the equilibrium of *cis trans* isomers [11]

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[5] H B ockmann *Z physi l Chem* 216 45 (1933)

[6] R Kuhn and C Grundmann *Be* 67 593 (1934)

[7] R Kuhn and H Brockmann *Z physiol Chem* 200 255 (1931)

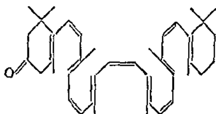
[8] O Ungnade *Chem Ztg* 63 9 (1939)

[9] R Willstätter and H H Escher *Z physiol Chem* 64 47 (1910) R Kuhn and H J Bielig *Ber* 73 1080 (1940)

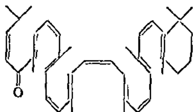
[10] L S Palmer *J Biol Chem* 23 261 (1915) *Carotinoids and Related Pigments* New York 1922 p 208

[11] E L Smith B I Stern and F E Young *Nature* 141 1 (1938)

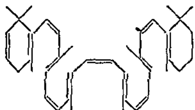
**Echinenone** Structure questionable! M p 192–193° 520 488 450 mμ ($CS_2$) E Lederer, *Compt rend* **201**, 300 (1935) E Lederer and T Moore, *Nature*, **137**, 996 (1936)



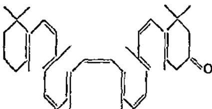
**Myxoxanthin** M p 168–169° 488 mμ ($CS_2$) 473 mμ ($CHCl_3$) 470 mμ ($C_2H_5OH$) 465 mμ (Light petroleum) I M Heilbron and B Lythgoe, *J Chem Soc* **1936**, 1376



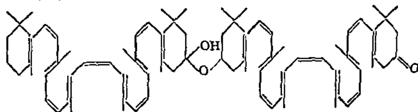
**Leprotene** Structure and provitamin A action questionable! M p 189–200° 517 479, 447 mμ ($CS_2$) 484, 452, 425 mμ (Benzene) C Grundman and Y Takeda *Naturwissenschaften*, **25**, 27 (1937) Y Takeda and T Ohta, *Z physiol Chem*, *258*, *6* *(1939)*, *262*, *168* (1939), **265**, 233 (1940), **267**, 171 (1941)



**Aphanin** Structure questionable! M p 180° 533 5, 494 mμ ($CS_2$) 504 474 mμ ($CHCl_3$) 505, 472 mμ (Benzene) 494 460 mμ (Petroleum) 507 5, 477 mμ (Pyridine) 491 5, 457 mμ (Methanol) J Tisher, *Z physiol Chem*, **251**, *109* (1938) A Scheunert and K H Wagner, *Ibid*, **260**, 272 (1939)

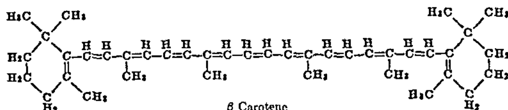



**Aphanicin** Structure questionable! M p 195° 533, 494 mμ ($CS_2$) 504, 474 mμ ($CHCl_3$) 505, 474 mμ (Benzene) 494, 462 mμ (Petro



leum) 507 5 478 mμ (Pyridine) 491 5 4 7 mμ (Methanol) A Scheunert and K H Wagner *Z physiol Chem* 260, 272 (1939)

The aliphatic part of the molecule is written in the manner of Ruzicka in the form of open rings This formula is not intended to indicate the true spatial arrangement of the aliphatic chain but to stress the relationship to similar compounds and to impress the memory Textbooks commonly use the long chain formula



β Carotene

Provitamins A are extremely sensitive to oxidation, autoxidation and light They are quite stable to heat in inert atmosphere

α Carotene is the only provitamin A with an asymmetric carbon atom and is, therefore optically active

The solubilities of the provitamins A are very similar They dissolve readily in chloroform carbon disulfide and benzene but more difficultly in petroleum ether They are practically insoluble in alcohol Cryptoxanthene due to the presence of a hydroxyl group in the molecule is somewhat soluble in alcohols All provitamins A are soluble in fats

## 5 Isolation

Crude preparations of mixtures of provitamins A are obtained by first separating the provitamins from the protein material to which they are usually bound in symplex form This is achieved by rapid heating[1] for example to 40–60° C or by reaction with invert soaps' such as lauryl dimethyl benzyl ammonium bromide[13] In the case of isolating provitamins A from vegetable oils an initial saponification with alcoholic potassium hydroxide is carried out In either case an extraction with organic solvents such as petroleum ether follows From the petroleum ether residue the crude mixture of carotenoids is either crystallized as such or a saponification may now be carried out in case this procedure was not employed in the beginning

[11] R Willstätter and H H Escher *Z physiol Chem* 64 47 (1910)
[1] R Kuhn and H J Bielig *Ber* 73 1080 (1940)

As stated, mixtures of carotenoids are obtained according to these procedures. The single compounds are obtained by methods which will be described separately for each provitamin.

α-Carotene seldom occurs as the only carotenoid in crude provitamin A preparations. It is usually accompanied by its isomer β-carotene, and sometimes by γ-carotene. Other carotenoids may be present. The total amount of α-carotene in crude carotene preparations varies between 0% and 40%. The highest concentration of α-carotene is found in red palm oil. Almost pure α-carotene has been found in leaves of tea from Formosa.[14] The progress in separation of the α-isomer from the other carotenoids can be followed by measuring the optical activity of the preparations. α-Carotene can be isolated, for example, by fractional precipitation with iodine in the form of a diiodo-α-carotene[15] followed by regeneration of α-carotene by means of sodium thiosulfate.[16] Fractional adsorption on aluminum oxide[17] or on fuller's earth[18] is a much better method. α-Carotene can be isolated quantitatively by chromatographic adsorption on calcium hydroxide or on magnesium oxide[19] from a solution of a crude preparation in petroleum ether. From a mixture of the α- and β-isomers an almost quantitative separation can be achieved by a single adsorption. A red layer of β-carotene and a lower more yellow layer of α-carotene,[20] are obtained.

β-Carotene is the easiest of all provitamins A to obtain in pure form. It is the only provitamin A in many plants. Its purity can easily be checked, since β-carotene is optically inactive, whereas the α-isomer has strong positive optical rotation. Small amounts of the γ-isomer can be separated by adsorption on calcium carbonate or calcium hydroxide. The separation of all provitamins A, and especially of β-carotene, from xanthophyll, a yellow chloroplast pigment of green leaves, can easily and quantitatively be achieved by distribution between petroleum ether and 90% methanol.[21] Carotenes remain almost entirely in the petroleum ether phase, while the xanthophyll passes into the methanol layer. Xanthophyll is 3,3'-dihydroxy-α-carotene.

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14 R. Yamamoto and [illegible] Muraoka, Sci. Papers Inst. Phys. Chem. Research (Tokyo), 19, 127 (1932).
15 R. Kuhn and E. Lederer, Ber., 64, 1349 (1931).
16 R. Kuhn and E. Lederer, Ibid., 65, [illegible] (1932).
17 R. Kuhn and E. Lederer, Z. physiol. Chem., 200, 246 (1931).
18 R. Kuhn and H. Brockmann, Ibid., 200, 255 (1931).
19 [illegible] P. Strain, [illegible] Chem., 105, [illegible] (1934).
20 P. Karrer and O. Walker, Helv. Chim. Acta, [illegible]
21 R. Willstätter and [illegible], [illegible]
[illegible]

**γ-Carotene** occurs only in very small quantities and is found together with β carotene Most technical carotene preparations contain about 0 001% of the γ isomer Occasionally, however, γ carotene occurs in larger quantities The carotenoids of *Gonocaryum pyriforme* fruit peels consist of 50–60% γ carotene [22] It can be separated from other carotenoids by chromatographic adsorption on aluminum oxide

**Cryptoxanthene** seems to occur frequently together with β carotene and other carotenoids, especially in red blossoms and fruits Especially high in cryptoxanthene are the calyx of *Physalis alkekengi* and *Physalis franchetii* (about 1/3 of the total pigment) and the pigments of yellow corn and of paprika In all these cases, cryptoxanthene does not occur in the free form and must be saponified before isolation Since cryptoxanthene has almost the same properties as β carotene fractionation does not result in a separation A number of different methods are available for separating β carotene from cryptoxanthene Distribution between petroleum ether and 90% methanol does not bring about any separation, distribution between petroleum ether and 95% methanol however, causes the crypto xanthene to pass slowly into the alcohol layer From a benzene solution of β carotene and cryptoxanthene only the latter is adsorbed on calcium carbonate The best method for the preparation of pure cryptoxanthene is the chromatographic adsorption on aluminum oxide

**Leprotene** has been isolated from bacilli namely from *Mycobacterium phlei* and from another bacillus of unknown species found in lepra diseases It is strongly adsorbed on aluminum oxide and can therefore be separated from the less strongly adsorbed β carotene

**Echinenone,** found in the sex glands of sea urchins occurs together with β carotene from which it can be separated by the chromatogram method

**Myxoxanthin** has been found in algae of the class *Myxophyceae* A convenient source is the fresh water species *Oscillatoria rubrescens* It has been separated from accompanying carotenoids, especially from β carotene by chromatographic adsorption on aluminum oxide, on which it is more strongly adsorbed than β carotene

**Aphanin** has been isolated from blue green algae of the Aphanizomenon family by chromatographic adsorption on aluminum oxide whereby it can be separated from other carotenoids for example β carotene which is less strongly adsorbed than aphanin

**Aphanicin** occurs together with aphanin and can be separated from

[22] A Winterstein Z physiol Chem 219 249 (1933)

this and from β carotene since it is more strongly adsorbed on aluminum oxide than either of the other accompanying carotenoids

## 6 Chemical Constitution

The provitamins A belong chemically to a special class of polyenes, the carotenoids They are characterized by a long aliphatic chain containing a continuous system of conjugated double bonds[23] which is responsible for their deep red color Many naturally occurring polyenes are known but only a few of them are provitamins

The chemical structure of all provitamins A is identical in their middle part, the symmetrical aliphatic chain of 18 carbon atoms with the continuous system of conjugated double bonds and four methyl groups constituting side chains They differ from each other by the structure of the groups on both ends of the aliphatic chain β Carotene has on both ends rings of the same structure as the ring of β ionone ($\Delta^5$ 1,1,5 trimethyl cyclo hexene) β Carotene, therefore, is called β,β carotene α Carotene (α,β carotene) has on one side a ring with the β ionone structure and on the other a ring with the α ionone structure (double bond in 4,5 position) γ Carotene has on one side a ring of the β ionone structure and on the other end no ring but the same number of carbon atoms as all other provitamins only in the form of an aliphatic chain (pseudo ionone structure), as in lycopene, the polyene from tomatoes In analogy with the other carotenes γ carotene may be called β lyco β carotene Cryptoxanthene is 3 hydroxy β,β carotene The methyl groups, which form side chains on the molecule, are in 1 5 position to each other, corresponding to the natural principle of the structure of most terpenes, which seem to be built up from isoprene residues by dehydrogenation [24] Only in the middle of the chains two methyl groups are in 1,6 position, thus dividing the molecule into two symmetrical halves [5]

Through catalytic reduction, β-carotene takes up 11 mols of hydrogen indicating the presence of 11 double linkages [26] Ozonization of β carotene gives geronic acid in the same yield as obtained by ozonization of two molecules of β ionone From α-carotene an equal amount of geronic and iso geronic acid was obtained γ-Carotene yielded only geronic acid, in an

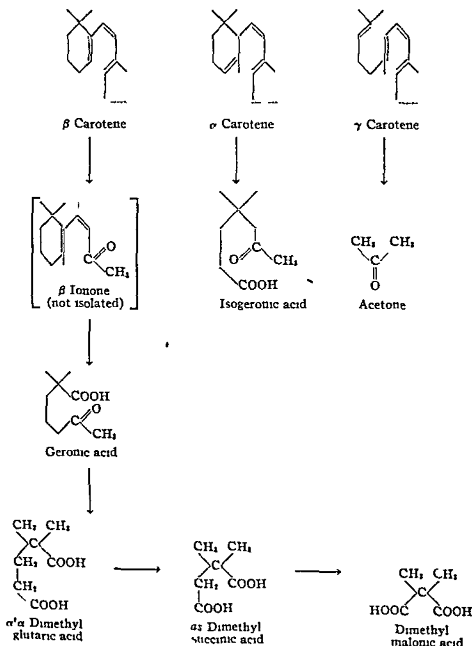
[23] R Kuhn and A Winterstein *Helv Chim Acta* 11 428 718 (1928) P Karrer and H Solomon *Ibid* 11 515 (1928)

[24] R Willstätter and W Mieg *Ann* 355 1 (1907) R Kuhn and A Winterstein *Helv Chim Acta* 11 430 (1928)

[25] P Karrer and co-workers *Helv Chim Acta* 13 1087 (1930) 15 1405 (1932)

[26] L Zechmeister L Cholnoky and V Vrabely *Ber* 61 566 1534 (1928)

amount corresponding to the presence of one ring of the $\beta$ structure Besides the geronic acid, ozonization of $\gamma$ carotene yields one molecule of acetone, split off from the aliphatic end of the molecule $\gamma$ Carotene has 12 double bonds one more than $\alpha$ or $\beta$ carotene Degradation of $\beta$ carotene with potassium permanganate gives four mols of acetic acid

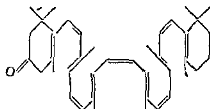


with chromic acid six mols This proves the presence of six methyl groups in the molecule Four of these, the ones giving acetic acid with permanganate, belong to the aliphatic chain, the other two came from methyl groups in the two rings at the ends

Permanganate oxidation of $\beta$ carotene yields $\alpha,\alpha$ dimethyl glutaric acid, *as* dimethyl succinic acid and dimethyl malonic acid

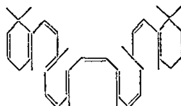
The presence of a secondary hydroxyl group in **cryptoxanthene** has been established by the presence of one active hydrogen atom and the formation of crystallized esters, for example, the acetate, which shows the same absorption spectrum as cryptoxanthene itself The esters cannot be adsorbed on calcium carbonate nor can they be extracted from a petroleum ether solution by means of 95% methanol The hydroxyl group, the exact position of which has not been determined, is assumed to occupy the carbon atom three in analogy to a number of other naturally occurring carotenoids especially lycopene

**Echinenone** is a monoketone of the probable formula $C_{40}H_{58}O(\pm H)$ Since it shows provitamin A activity, one part of the molecule is assumed to contain the $\beta$ ionone structure It is perhaps a dehydro cryptoxanthene of the following structure




**Myxoxanthin** is a monoketone of the formula $C_{40}H_{54}O$ and forms an oxime By hydrogenation 12 mols of hydrogen are taken up and 13 mols are absorbed by hydrogenation of the oxime indicating the presence of 12 ethenoid linkages Since myxoxanthin possesses growth promoting properties one part of the molecule probably has the $\beta$ ionone configuration Myxoxanthin is, therefore monocyclic and must be classified as a derivative of $\gamma$ carotene By reduction of the keto group to the alcohol group with aluminum isopropoxide, myxoxanthol is obtained which is spectroscopically identical with $\gamma$ carotene

**Leprotene** probably has the constitution of a dehydro $\beta$ carotene Since it contains two hydrogen atoms less than $\beta$ carotene but exhibits the same absorption spectrum characteristics, the following formula is most probable



Aphanin is a monoketone of the formula $C_{40}H_{54}O$ The presence of the keto group has been established by the formation of a crystallized oxime From the absorption spectrum of this oxime, which is the same as that of aphanin it is concluded that the keto group is not in conjugation with the system of double bonds The negative result of an isopropylidene determination excludes the possibility of an open chain structure at one end of the molecule Catalytic hydrogenation indicates the presence of 12 double bonds one of which belongs to the keto group

Aphanicin is a dicarotenoid consisting of two molecules of aphanin linked together by an oxygen bridge The analysis conforms with the empirical formula $C_{80}H_{106}O_3$ The presence of a keto group was ascertained by the formation of an oxime and the molecular weight is indicated by the physiological effect of aphanicin as provitamin A

## 7 Synthesis

None of the provitamins A has been synthesized as yet

## 8 Industrial Methods of Preparation

The technical isolation of provitamins A from plant material is simple compared with the difficulties of manufacturing some of the other vitamins Two different starting materials are chiefly used in industry fresh plant material, such as carrots nettle lucerne grass etc or vegetable oils among which red palm oil is one of the richest sources of provitamins A

The main difficulty in extracting carotene from fresh plant material is the presence of large amounts of water which hinders the extraction with organic solvents Fresh plant material must therefore be dried which process is always attended by some loss of provitamins The best results are obtained by a rapid short drying process preferably at low temperature and under vacuum Another method is the addition of water binding agents to the freshly ground plant material As water binding agents sodium sulfate sodium carbonate anhydrous calcium sulfate etc, are recommended Another method which however, proved to be too ex

pensive, is the extraction of fresh plant material with alcohol, which takes up all the water, but leaves the carotenes undissolved

The extraction of carotene from the dried material is brought about by organic solvents, such as benzene, petroleum ether, acetone, etc These solvents dissolve, besides the carotene, all fats present In order to obtain carotene free from other materials, and especially free from fat a saponification of the extract must be carried out An alternative method is the saponification of the total plant material before extraction In most cases this latter procedure is too expensive Since carotenes are somewhat sensitive to alkali, especially at higher temperatures (rearrangement of double bonds), the use of large excesses of alkali is carefully avoided

The juice of plant material, for example, of carrots is also used as starting material for carotene manufacture The juice contains large amounts of protein materials, which adsorb the carotene when coagulated The coagulation is carried out by the different known procedures, for example, by heating, by acids, by precipitation with lead acetate, magnesium chloride, etc The precipitates are in turn extracted with organic solvents to isolate the carotenes

It is not always of technical importance to prepare crystalline carotenes Fat concentrates or dried grass and similar products are often used for food supplements

The preparation of pure, single provitamins A is seldom carried out technically, although two different methods can be applied, namely, the precipitation of $\beta$ carotene with iodine and the separation by chromatographic adsorption

## 9 Biogenesis

Little actual knowledge exists about the biogenesis of provitamins A although this problem has attracted widespread attention As far as is known, only plants and certain microorganisms have the power of synthesizing provitamins A Their constitution leads to the supposition that the building units of the provitamins are the same as those of most terpenes and polyterpenes All these compounds appear to be built up from a simple, five carbon atom containing substance of the general formula of isoprene Thus, all provitamins can be regarded as resulting from a condensation[27] together with a simultaneous dehydrogenation[28] of isoprene molecules Actually, however, neither isoprene nor any other similar

[27] R Willstätter and W Mieg *Ann* 355 1 (1907)
[28] R Kuhn and A Winterstein *Helv Chim Act* 11 427 (1928)

compound has ever been found in plants This can be explained of course on the basis that these compounds are anabolic intermediates which are not stored as such but are used immediately for the synthesis of other compounds Besides isoprene other compounds have been suggested as potential intermediates Among these, β methyl crotonaldehyde,[29] levulinic acid[30] and methyl vinyl ketone are outstanding because of their obvious ability to condense with other molecules

Without any doubt carotenoids are built up by total synthesis in plant tissues It is questionable however whether or not the provitamins A are synthesized according to the above outlined scheme The possibility that they might be formed by dehydrogenation of compounds of similar structure has also been discussed Thus for example phytol[31] or a related compound[32] may serve as a starting material

The synthesis of provitamins A appears to be catalyzed by light Generally speaking the carotene content of plants grown in light is several times higher than the content of plants raised in the dark Tissues of plants grown in the absence of light contain compounds which are believed to be precursors of the provitamins A The former however are essentially colorless (perhaps saturated compounds) and differ from the provitamins in many other respects such as water solubility [33] On the other hand, it appears remarkable that carrot roots for example contain a more abundant amount of provitamins A than the leaves It might be assumed of course that these provitamins were synthesized in the leaves and transported into the roots This hypothesis is however in contradiction to the general belief that plants do not have a mechanism for the transportation of fat soluble, that is non water soluble materials The additional hypothesis might then be considered that a water soluble precursor is synthesized in the leaves transported to the roots and there transformed (possibly by dehydration or dehydrogenation) into provitamins On the other hand the possibility cannot be excluded that these provitamins A are totally synthesized in the roots even in the absence of light

## 10 Determination

The problem of determining quantitatively the amount of provitamins A in natural materials is quite difficult In the analysis of plant material the

[29] H v Euler and E Klussmann *Sv nsk Kem Tid* 44 198 (193 )
[30] H Emde *Helv Chim Acta* 14 881 (1931)
[31] L Zechmeister and L Cholnoky *Ann* 465 88 (19 8)
[32] P Karrer H Helfenstein H Wehrl and A Wettstein *Helv Chim Acta* 13 1084 (1930)
[33] R Willstätter and A Stoll *Untersuchungen über die Assimilation der Kohlensäure* Berlin 1918, p 134

problem is somewhat simplified by the absence of vitamin A, which prevails in animal tissues. The methods used for the analysis of provitamins A in the presence of vitamin A are described on page 80.

The only exact method for the provitamin A assay is the biological method using pure β carotene as a standard reference. This method gives average values of the total carotenoid mixture expressed as β carotene equivalent (see page 82).

Approximate provitamin A values can be obtained by either physical or chemical methods. They involve a separation of the provitamins A from other carotenoids present, especially xanthophyll (lutein) and other phytoxanthins. Such a separation is based on the fact that α, β and γ carotenes are hydrocarbons and the phytoxanthins are oxygenated carotene derivatives, that is, alcohols and ketones. They show different solubility characteristics and can therefore be separated to a certain extent. These separations do not bring about a separation of provitamins A which contain hydroxyl or keto groups from non provitamin A carotenoids with such functional groups. Fortunately, the average provitamin A analysis in foodstuffs can disregard all provitamins A other than the carotenes proper, since they are found only infrequently.

The actual assay procedure involves, first, the separation of the provitamin A from the protein to which it is bound in animal and plant materials. This is accomplished either by heating to 40–60° C or by the addition of invert soaps such as lauryl dimethyl benzyl ammonium bromide.[34] This is followed by an extraction from the natural source, either before or after a saponification.[*] The alcohols, ethers, petroleum ether, hexane, acetone, pyridine and mixtures, such as petroleum ether and methanol or petroleum ether and acetone, have been recommended as solvents. The saponification is carried out with potassium hydroxide in alcohol, preferably at room temperature, although the application of heat is recommended when entire tissues are to be saponified. Saponification with aqueous potash has also been advocated. The main object is to bring the pigments in the saponified form into a solution of petroleum ether. This solution is then washed with dilute alkali and water to effect a separation from the chlorophyll. The actual separation of the carotenoids is then accomplished by shaking the petroleum ether solution with 92% methanol (by volume). The hydrocarbons remain in the petroleum ether layer while the oxygenated xanthophylls go into the methanol phase.[3] After repeated extractions, the pe-

[34] R. Kuhn and H. J. Bielig, *Ber.*, 73, 1080 (1940).
[3] R. Willstätter and A. Stoll, *Untersuchungen über Chlorophyll*, Berlin, 1913.

troleum ether solution is used for direct determinations [36] Thus the total amount of carotenes can be determined spectrophotometrically and is usually expressed in terms of $\beta$ carotene Determinations are usually made of only one wave length using the 450 m$\mu$ band of $\beta$ carotene for which $E^{1\%}_{1\,cm}$ = 2500 (in petroleum ether) It has also been suggested that the provitamins A be determined colorimetrically by comparison with the color of standard dye solutions for example, of azobenzene of known concentrations[37] or of a 0 1% solution of $K_2Cr_2O_7$ [38, 39]

Another method is to develop a blue color according to the method of Carr and Price (see page 78) and to determine the color by spectrophotometric methods

Qualitative estimations for the presence of individual provitamins A can be made by using the chromatographic adsorption technique for the separation from other carotenoids The methods used are the same as described for the preparation of the individual provitamins A The principle of preferential adsorption has also been developed for the quantitative estimation of carotenes Thus a specially prepared magnesium carbonate has been developed to absorb xanthophyll but not carotene [40, 41] and to absorb lycopene but not carotene [41, 42]

## CONVERSION OF PROVITAMINS A INTO VITAMINS A

Most substances ingested by human beings and by animals which bring about vitamin A action are not identical with the vitamins A found in animal organisms They are provitamins A In 1919 Steenbock[43] found that A avitaminosis in rats can be cured with fresh green plant materials and that the healing action corresponded to the amount of carotene present This result could not be repeated until it was demonstrated ten years later[44] that the avitaminotic animals needed also vitamin D to regain health It was furthermore discovered that the vitamin A content of the liver of

[36] Tentative method of the Association of Official Agricultural Chemists for the determination of carotene *J Assoc Official Ag Chem* 22 79 (1939)

[37] P Karrer and K Schöpp *Helv Chim Acta* 15 745 (1932) R Kuhn and H Brockmann *Z physiol Chem* 206 41 (1932)

[38] H R Guilbert *Ind Eng Chem Anal Ed* 6 452 (1934)

[39] V E Munsey *J Assoc Official Ag Chem* 22 664 (1939)

[40] G S Fraps and A R Kemmerer *Ibid* 22 190 (1939)

[41] G S Fraps A R Kemmerer and S M Greenberg *Ibid* 23 4 (1940)

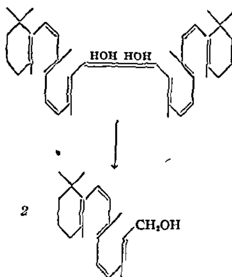
[42] G S Fraps A R Kemmerer and S M Greenberg *Ibid* 23 6 (1940)

[43] H Steenbock *Science* 50 352 (1919) H Steenbock and P W Boutwell *J Biol Chem* 41 81 (1920) H Steenbock M T Sell E M Nelson and M V Buell *Ibid* 46 3 (1921) H Steenbock and E G Gross *Ibid* 51 63 (19 )

[44] B v Euler H v Euler and P Karrer *Helv Chim Acta* 12 278 (1929) H v Euler V Demole P Karrer and O Walker *Ibid* 13 1078 (1930)

rats decreased[45] rapidly and finally disappeared completely when the animals were kept on a vitamin A free diet After the addition of carotene to the diet, the vitamin A content of the liver increased again rapidly

At the same time, vitamin A was isolated from liver oils and its formula was established [46] Vitamin A contains just half as many carbon atoms as carotene Theoretically, two mols of vitamin A *must split out two mols* of water to become β carotene




*The hypothesis was then advanced that β carotene is split in the organism* with the addition of two molecules of water to yield two molecules of vitamin A This theory was strongly supported by the difference in the quantitative physiological efficacy of α and β carotene There exist however trustworthy observations[47] which indicate that even under optimum conditions an unsymmetrical fission of provitamin A occurs which in the case of β carotene yields a maximum of only one mol of vitamin A plus other decomposition products This is in agreement with the observed potency of provitamin A in many cases (see page 75)

The mechanism of the conversion of provitamins A to vitamins A is not known It is assumed that the conversion is effected by an enzyme called carotenase. It is not known with certainty in which organ the conversion takes place but much evidence exists that it is in the liver [48] Furthermore the pancreas appears to be involved since in human beings with

[45] T Moore *Biochem J* 24 691 (1930)

[46] P Karrer R Morf and K Schöpp *Helv Chim Acta* 14 1036 1431 (1931)

[47] S W F Underhill and K H Coward *Biochem J* 33 589 (1939) *H v Euler P Karrer and A Zubrys Helv Chem Acta* 17 24 (1934)

[48] *H S Olcott and D C McCamm J Biol Chem* 94 185 (1931) B Ahmad *Biochem J* 25 1195 (1931) J I Rea and J C Drummond *Z Vitaminforsch* 1 177 (1932)

tinal tract contains smaller amounts Considerable quantities are present in the retina and the corpora lutea small amounts in lungs and kidney[69] Vitamin A is present also in egg yolks (one hen egg contains about 20 U S Pharmacopoeia Units),[70] milk (3 U S Pharmacopoeia Units per gram)[71] and milk products (butter, 50 U S Pharmacopoeia Units per gram) Colostrum of man and of cows is from two to ten times richer in vitamin A than the milk Stored fats contain small amounts of vitamin A, especially during pregnancy

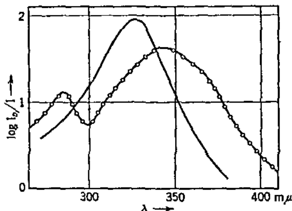



Fig 3 — Absorption spectra of vitamin A (——) and of vitamin $A_2$ (o-o-o) (A E Gillam I M Heilbron W E Jones and E Lederer )

tamin A occurs both as free alcohol and predominantly in esterified in the animal organism[72] Vitamin A palmitate has been isolated the liver oil of *Stereolepis ishinagi*[73] and from cod liver oil[74] in the form addition product with maleic anhydride The free vitamin A has eparated[75] from its esters by molecular distillation By the use of ne method it has also been shown that different esters are present in [illegible]ver oil and cod liver oil

*B ochem J* 25 7 (1931)
and E Klussmann *B ochem Z* 219 215 (1933)
n and I M He[illegible] *B ochem J* 24 880 (1930) T Moore *B ochem J* 26 1

and E I [illegible] *J Pharm* 1 539 (19[illegible]) J Reti *Compt rend s c biol*

*Papers In* [illegible] [illegible]*em Research (Tokyo)* 26 87 (1935) K Kawak m *Ib d*

*Ind I* [illegible] 29 968 1107 (1937)
*I Ch m* [illegible] (1938)

## 12 Occurrence

Vitamin A occurs only in the animal organism, it has not been found in plants

The most important source of vitamin A is liver oils because the organism stores most of the excess vitamin A in the liver The amount of vitamin A in the liver varies greatly, according to the type of food consumed, general living conditions, season etc Fish liver oils from cod halibut, tuna, etc, contain vitamin A in fair amounts Especially rich in vitamin A are the liver oils from *Hippoglossus hippoglossus*, *Scombresox saurus*, *Rhombus maximus* and from the Japanese fish, *Stereolepis ishnagi* which contain from 200 to 2000 times as much vitamin A as the common cod liver oil

TABLE I

DISTRIBUTION OF VITAMIN A IN VARIOUS FISH LIVER OILS

| Source of oil | Zoological name | Potency I U per gram |
|---|---|---|
| Haddock | *Gadus alglefinus* | 65 |
| Cod | *Cadus morrhua* | 600 |
| Striped bass | *Roccus lineatus* | 4 500 |
| Jack smelt | *Atherinopsis californiensis* | 10 000 |
| Albacore | *Cermo alalunga* | 18 000 |
| Grouper | *Epinephelus morio* | 25 000 |
| Boston mackerel | *Scomber scombrus* | 30 000 |
| Barracuda | *Sphyraena argentea* | 40 000 |
| Skipjack tuna | *Katsuwonus pelamis* | 40 000 |
| Yellowtail | *Seriola dorsalis* | 50 000 |
| White sea bass | *Cynoscion nobilis* | 50 000 |
| Red snapper | *Lutianus campechanus* | 60 000 |
| Totuava | *Eriscion macdonaldi* | 60 000 |
| Bluefin tuna | *Thunnus thynnus* | 60 000 |
| Yellowfin tuna | *Neothunnus macropterus* | 70 000 |
| Pacific mackerel | *Pneumatophorus diego* | 80 000 |
| Bonito | *Sarda chiliensis* | 120 000 |
| Cabrilla pinta | *Epinephelus analogus* | 170 000 |
| Swordfish | *Xiphias gladius* | 250 000 |
| Ishnagi | *Stereolepis ishnagi* | 300 000 |
| Black sea bass | *Stereolepis gigas* | 600 000 |

Besides the liver, other organs contain some vitamin A the most important of which are the pyloric ceca of a number of fish species These contain an amount equal to that found in the liver [68] The rest of the intes

[68] J R Edisbury R A Morton C W Simpkins and J A Lovern *J Biol Chem* 32 118 (1938)

tinal tract contains smaller amounts Considerable quantities are present in the retina and the corpora lutea, small amounts in lungs and kidney[69] Vitamin A is present also in egg yolks (one hen egg contains about 20 U S Pharmacopoeia Units),[70] milk (3 U S Pharmacopoeia Units per gram)[71] and milk products (butter, 50 U S Pharmacopoeia Units per gram) Colostrum of man and of cows is from two to ten times richer in vitamin A than the milk Stored fats contain small amounts of vitamin A especially during pregnancy

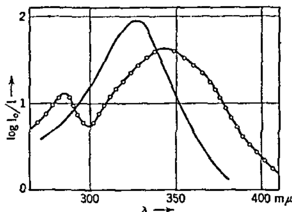



Fig 3 — Absorption spectra of vitamin A (——) and of vitamin $A_2$ (o-o-o) (A E Gillam I M Heilbron W F Jones and F Lederer )

Vitamin A occurs both as free alcohol and predominantly in esterified form in the animal organism[72] Vitamin A palmitate has been isolated from the liver oil of *Stereolepis ishnagi*[73] and from cod liver oil[74] in the form of an addition product with maleic anhydride The free vitamin A has been separated[75] from its esters by molecular distillation By the use of the same method it has also been shown that different esters are present in halibut liver oil and cod liver oil

[69] T Moore *Biochem J* 25 ? 5 (1931)
[70] H v Euler and E Klussmann *Biochem Z* 219 215 (1933)
[71] R A Morton and I M Heilbron *Biochem J* 24 860 (1930) T Moore *Biochem J* 26 1 (193?)
[72] A L Bacharach and E L Smith *Quart J Pharm* 1 539 (19 ?) 1 Ret *Compt rend soc biol* 120 577 (1935)
[73] Hamano *Sc Papers Inst Phys Chem Research* (Tokyo) 26 87 (1935) K Kawakami *Ibid* 26 7 (1935)
[74] K C D Hickman *Ind Eng Chem* 29 968 1107 (1937)
[75] A O Tischer *J Biol Chem* 125 475 (1938)

## 13 Properties

Vitamin A crystallizes from methanol in pale yellow plates, containing solvent of crystallization and melting at 8° C [77] A purer form of vitamin A crystals, melting at 63–64° C is obtained from ethyl formate or from propylene glycol [76] Vitamin A was found to distill at 137–138° C at $10^{-4}$ mm [78] but later workers found the material to evaporate at 120–125° C at $5 \times 10^{-3}$ mm [79] The natural vitamin A esters distill from 200–240° C at $10^{-4}$ mm The absorption spectrum of vitamin A in the ultraviolet shows a sharp band at the wave length 328 mμ (Fig 3) with an extinction coefficient $E^{1\%}_{1\,cm} = 1725$ [77] The biological activity is 4 300 000 International Units per gram [80]

Vitamin A is sensitive to oxidation and autoxidizes readily It is quite heat stable in inert atmosphere and is readily stabilized in solution in oil The esters of vitamin A are more stable than the free substance Vitamin A is destroyed by ultraviolet light and is optically inactive Vitamin A crystals appear to be isotropic Vitamin A is soluble in most organic solvents but is insoluble in water It has, therefore, been classified as a member of the fat soluble vitamins

## 14 Isolation

Vitamin A can be isolated from the unsaponifiable parts of animal fats, especially liver oils Three different methods have been used for the isolation, namely, chromatographic adsorption, vacuum distillation and fractional crystallization at low temperatures

The use of the chromatographic adsorption method[81] brought about the first preparation of almost pure material The liver oils of *Hippoglossus* and of the mackerel *Scombresox saurus* were saponified and the unsaponifiable part freed from sterols by cooling to −70° C The non crystallized material possesses the vitamin A activity By adsorption on aluminum oxide followed by differential adsorption on calcium hydroxide a concentrate was obtained, which by repeated adsorption could not be further

[76] J G Baxter and C D Robeson *Science* 92 204 (1940)
[77] H N Holmes and R E Corbet *J Am Chem Soc* 59 2042 (1937)
[78] I M Heilbron R N Heslop R A Morton E T Webster J L Rea and J C Drummond *Biochem J* 26 1178 (1932) F H Carr and W J Jewell *Nature* 131 92 (1933)
[79] K C D Hickman *Ind Eng Chem* 29 968 1107 (1937)
[80] J G Baxter P L Harris K C D Hickman and C D Robeson *J Biol Chem* 141 991 (1941)
[81] P Karrer R Morf and K Schöpp *Helv Chim Acta* 14 1036 1431 (1931)

purified [8] It consisted of a viscous yellow oil which was used for the successful determination of the chemical structure

The disadvantage of this method lies in the fact that due to the sensitivity of the vitamin A molecule partial destruction occurs during the adsorption procedure Rearrangements of the double bonds and slight oxidation might be the chief reasons for the change of the compound

By the use of the vacuum distillation process vitamin A concentrates have been obtained[83, 84, 8, 86] of the same properties as obtained by the use of the chromatographic adsorption method A decided improvement is the use of the short path high vacuum distillation which has resulted in the isolation of vitamin A and its esters in relatively pure form and has enabled the determination of exact boiling points and distillation characteristics This has been accomplished by the use of the cyclic molecular still and certain adjuncts such as constant yield oils and pilot ' dyes The saponified vitamin concentrate is dissolved in a mixture of neutral residue oil and constant yield oil and is distilled in stepwise fashion over long ranges of temperatures The concentration of vitamin A in the distillates is plotted against temperature and results in an elimination curve ' with a distillation maximum on the temperature axis Using this method vitamin A is shown to distill at 123° as compared with celanthrene red distilling at 125° C [87]

Vitamin A was finally crystallized[88] by the use of fractional freezing and cold filtration after the isolation of the unsaponifiable material Vitamin A crystals are best obtained by crystallization from ethyl formate or from propylene oxide [89] From methanol vitamin A crystallizes with solvent of crystallization Pure vitamin A melts at 63 64° C [8]

## 15 Chemical Constitution

The constitution of vitamin A has been determined mainly by Karrer who suggested the following structure ,

[8] P Karr r nd R Morf *Helv Chim Acta* 16 6 (1933)

I M Heilbron R N Heslop R A Morton F T Web ter J I Rea and J C Drumm n l *Biochem J* 26 1178 (1932)

[83] F H Carr and W J Jewell *Nature* 131 92 (1933)

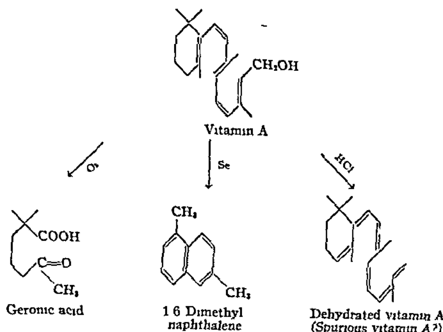
[84] J C Drummond H J Channon and K H Coward *Bi chem J* 19 1047 (192 )

[85] Vitamin A prepar d accord ng to th s method was used in 1935 to set up a provision l tan lard E M Hume and H Ch ck *Med Resea ch Cou c l Rept IV The Sta dardisat on and Estimat on of Vitamin A* 193

[86] K C D H ckman *Ind Eng Chem* 29 968 1107 (1937) D H Killeffer *Ib d* 29 368 (1937) N D Embree *Ibid* 29 915 (1937) J G B xter P I Gray and O A Tinker *Ibid* 29 1112 (1937)

[87] H N Holmes and R F Corbet *J Am Chem Soc* 59 2042 (1937)

[88] J G Baxter and C D Robeson *Science* 92 202 (1940)




The empirical formula is $C_{20}H_{30}O$ Various crystallized esters for example the beta naphthoate m p 76° C the anthraquinone β carboxylic acid ester m p 126° C [90] the acetate m p 56–58° C, the palmitate m p 26–28° C and the disuccinate m p 73–75° C, have been prepared and indicate the alcoholic function of the oxygen Molecular weight determinations proved to be extremely difficult to carry out due to rapid changes of the vitamin in solution The best experimental values obtained show an average molecular weight of 294 but it seems probable that the correct weight must be a little less [91] (Karrer's formula calls for 286)

Oxidation of vitamin A with ozone yields geronic acid which proves the presence of the β ionone ring The methyl groups in the side chain are converted into acetic acid by oxidation with permanganate in analogy to the results obtained by the oxidation of the provitamins A Dehydrogenation of vitamin A with selenium forms 1 6 dimethyl naphthalene by closing a second ring and splitting off two methyl groups and part of the side chain [92] By the action of mineral acids on vitamin A a transformation occurs, which is indicated by a change of the absorption spectrum It has been suggested[93] that under the influence of acids a second ring is formed

[90] S Hamano *Sci Papers Inst Phys Chem Research* (Tokyo) 28 69 (193 ) 32 44 (1937) T H Mead *Biochem J* 33 589 (1939)

[91] *H R Bruins J Overhoff and J L Wolff Biochem J* 25 430 (1931) H N Holmes and R E Corbet *J Am Chem Soc* 59 2042 (1937)

[92] I M Heilbron R A Morton and E T Webster *Biochem J* 26 1194 (1932)

[93] J R Edisbury A E Gillam I M Heilbron and R A Morton *Ibid* 26 1164 (1932)

It is more plausible that a dehydration may take place A compound apparently identical with dehydrated vitamin A and often referred to as spurious vitamin A has also been found to be present as a normal constituent of fish liver oils (The absorption maxima occur at 350 368 and 379 mμ [94])

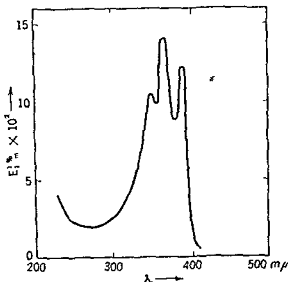



Fig 4 —Absorption spectrum of anhydro vitamin A (N D Embree)

Vitamin A contains a continuous series of five conjugated double bonds Maleic and citraconic anhydrides form addition products of unknown constitution [95] By catalytic hydrogenation of vitamin A five mols of hydrogen are absorbed The reaction product perhydro vitamin A has been prepared synthetically according to the following scheme[96] β Ionone and bromo ethyl acetate were condensed with zinc according to Reformatsky forming β ionylidene ethyl acetate (II) By catalytic hydrogenation the double bonds were saturated and by reduction with sodium and alcohol according to Bouveault Blanc the ester group was converted into an alcohol

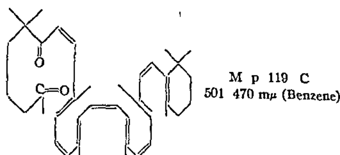
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[94] D C Castle A L Gillam I M Heilbron and H W Thompson *Biochem J* 28 170 (1934) I M Heilbron R N Heslop R A Morton J L Rea and J C Drummond *Ibid* 26 11 8 (193 ) H Pritchard H Wilkinson J R Edisbury and R A Morton *Ibid* 31 58 (1937) N D Embree *J Biol Chem* 128 187 (193 )

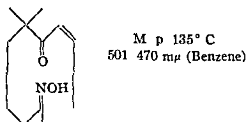
[95] S Hamano *Sc Pap Inst Phys Chem Research (Tokyo)* 25 536 538 (1934) 26 8 (1935) K Kawakami *Ibid* 26 77 (193 ) / Nakamiya *Ibid* 27 4 (193 )

[96] P Karrer and R Morf *Helv Chim Acta* 16 557 6 (1933)

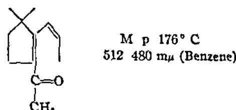
in a daily dose of 5 γ Carotene iodide is also active in a daily dose of 40 γ probably due to a regeneration of carotene [141]




(I)
β Semi-carotenone



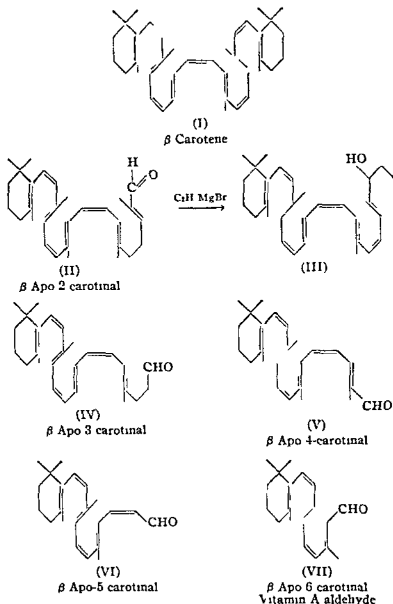

(II)
β Semi-carotenone monoxime




(III)
β Dehydro semi carotenone

Some of the degradation products of β carotene are biologically active whereas the corresponding derivatives of α carotene appear to be inactive By mild oxidation of β carotene with permanganate a series of aldehydes was obtained by fission of various double bonds Thus, the main reaction product, β apo 2 carotinal[142] (formula (II) on page 77) is obtained by an oxidation of the double bond in the aliphatic chain of the molecule adjacent to one ring This compound as well as its oxime proved to be active in a daily dosage of 5 γ [143] The vitamin A produced from β apo 2 carotinal in rats resembles in all characteristics the vitamin A or axerophthol [144] In other words this degradation product of β carotene is apparently not a vitamin A but a provitamin A By reduction with aluminum isopropylate, β apo 2 carotinal can be converted into the corresponding alcohol β apo 2 carotinol, which compound probably also is a provitamin A By condensation of β apo 2 carotinal with ethyl magnesium bromide, a tertiary alcohol of the formula (III) is produced, which is also active [145]

[141] P Karrer and M Rydbom *Ber* 62 2445 (1929) T Moore *Lancet* 1929 II 219
[142] P Karrer and U Solmssen *Helv Chim Acta* 20 682 (1937)
[143] H v Euler P Karrer and U Solmssen *Ibid* 21 211 (1938)
[144] H v Euler G Günther M Malmberg and P Karrer *Ibid* 21 1619 (1938)
[145] P Karrer A Ruegger and A Geiger *Ibid* 21 1171 (1938) H v Euler G Gunther M Malmberg and P Karrer *Ibid* 21 1619 (1938)




(I)
β Carotene

(II)
β Apo 2 carotinal

(III)

(IV)
β Apo 3 carotinal

(V)
β Apo 4-carotinal

(VI)
β Apo-5 carotinal

(VII)
β Apo 6 carotinal
Vitamin A aldehyde

The next lower homolog of β apo 2 carotinal is β apo 3 carotinal (formula (IV)) which is present in the oxidation products in small quantities, but which has not been isolated β Apo-4 carotinal has been obtained however in the form of its crystallized oxime which is biologically active The corresponding alcohol has also been prepared The next lower homolog would be β apo 5 carotinal the alcohol of which has the formula which has tentatively been assigned to vitamin $A_2$ This compound has not yet

been isolated from the oxidation products of carotene, although lower homologs are present The absorption spectra of the series of ' homolog alcohols of axerophthol indicate a constant shift of the maxima toward higher wave lengths with an increasing number of double bonds present (see Fig 5) From these data it appears questionable that vitamin $A_2$ has the formula of a $\beta$ apo 5 carotinol

## 20 Determination

### (a) *Chemical Methods*

A great number of different color reactions have been proposed for the determination of vitamin A These color tests are based on three different types of reactions reaction with phenols, with acids, and with inorganic chlorides, which in aqueous solution show an acidic reaction

The method of Carr and Price[148] is the only one in general use A 20–25% solution of antimony trichloride in chloroform is added to a solution of vitamin A in chloroform To insure greatest possible accuracy in the determination the antimony trichloride reagent should be free of any traces of ferric chloride [146] A blue color develops which shows its maximum after 10 seconds and then immediately begins to fade The color is measured in a colorimeter for example, in the Lovibond tintometer (visual or photoelectric[147] observation), or by spectrophotometric analysis, The values obtained may be checked against standardized solutions of copper sulfate[149] and cobalt nitrate or against certain color filters The value of a 20% solution of the vitamin containing material in chloroform is called a Lovibond Unit For international use a 2% solution is recommended The values obtained by this latter method are called "C L O (cod liver oil) Units Various other manners of expressing the color value have been recommended for example the Carr Price value,[148] the Moore[150] blue units and the Dann Evelyn[151] L 620 mμ value A noteworthy variation is the Anderson Nightingale[15] dilution test which consists in diluting the material to be analyzed for its vitamin A content with chloroform until the blue color is just visible (For comparison of different units see also page 83 )

[146] J R Edisbury *Analyst* 65 484 (1940)
[147] R L McFarlan J W Reddie and E C Merrill *Ind Eng Chem Anal Ed* 9 3 4 (1937)
[148] F H Carr and F A Price *Biochem J* 20 498 (1926)
[149] H Brockmann and M L Tecklenburg *Z physiol Chem* 221 117 (1932)
[150] T Moore *Biochem J* 23 1267 (1929) 31 155 (1937)
[151] W J Dann and K A Evelyn *Ibid* 32 1008 (1938)
[152] A Andersen and I Nightingale *J Soc Chem Ind* 48 139T (1929)

The values obtained with the Carr Price method for vitamin A from liver oils are in fairly good agreement with the results of biological tests Crude liver oils contain substances which interfere with the color reaction [154] and should therefore be saponified under anaerobic conditions and in the ab sence of sunlight before testing Some naturally occurring acids decrease the value [155] Some carotenoids for example cyclized vitamin A increase the value It is possible, however to differentiate by the use of selective filters between the colors produced by vitamin A and by carotenes by meas uring the absorption the maximum being at 620 mμ for vitamin A and at 590 mμ for carotene The maximum for vitamin $A_2$ lies at 693 mμ Im purities and mixtures of vitamins A and carotenes may change the wave length of the maximum of absorption [156] Visually the colors produced by carotene and vitamin A can be differentiated since the blue obtained from carotene persists without fading while the blue from vitamin A fades in two to five minutes A mixture is indicated when the color fades rapidly at first but remains constant later on A red color is caused by the presence of sterols [153] The Carr Price color reaction cannot be used for the determination of biologically active material in synthetic vitamin A prepa rations

Antimony trichloride causes a change in the chemical structure of the carotenoids the nature of which is unknown The reaction product is biologically inactive [157]

A number of modifications of the Carr Price test have been proposed for example the addition of a 0 5% pyrocatechol [158] the addition of arsenic trichloride and hydroqui none [159] determination at low temperature [160] determination of the blue value minus the yellow value [161] etc [162] [163]

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Y Raoul a d P Meun er *J pha m chim* 29 112 (1934)

E R Norris and A E Church *J Biol Chem* 85 477 (1929) 87 131 (1930) 89 421 (1930) F I Sm th and V Hazley *Biochem J* 24 1942 (1930) K H Coward F J Dyer R A Morton and J H Gaddum *Ib d* 25 1102 (1931) R S Morg n *Ib d* 26 1144 (1932)

[154] A Emmerie *Natu e* 131 364 (1933) *Acta B evia Nee land Phys ol Pha macol Microbiol* 2 158 (1933) *Prcc R y Soc Amsterdam* 35 1347 (1932)

[155] H v Euler P Karrer E Kluss m nn and R Morf *Helv Chim Acta* 15 50 (193 ) H Brock mann nd M L Tecklenburg *Z physiol Chem* 221 117 (1933) I M Heilbron A F G llam and R A Morton *B ochem J* 25 1352 (1931) M v n Eekelen A Emmerie H W Jul us and K ( Wolff *Acta Brev a Neerla d Physiol Pharmacol Mic ob ol* 1 8 (1931)

[156] A L Gillam I M Heilbron R A Morton and J C Drummond *B ochem J* 26 1174 (193 )

[157] J Rosenth l and J Erdély *Ibid* 28 41 (1934)

[158] G Gutz t *Arch sci phys nat* 9 155 (1927)

[159] E R Norris and co-worker *J B ol Chem* 85 4 7 (1929) 89 421 (1930) *J N trit on* 5 49 (1932)

[160] R S Morgan *Biochem J* 26 377 (193 )

[161] F I Smith nd V H zley *Ib d* 24 1942 (1930) 27 17 (1933)

[162] T Moore *Ib d* 24 692 (1930)

The Carr-Price method uses the principle of developing a color of vitamin A with an inorganic chloride in anhydrous solvents The same principle is used in tests with aluminum chloride arsenic chloride[164] ferric chloride or stannic chloride[165] The principle of using acids to produce a measurable color with vitamin A has been recommended Sulfuric acid phosphoric acid[166] chloric acid,[167] molybdenum phosphotungstic acid[168] and trichloroacetic acid[169] are used either alone or in combination with the phenol principle, to produce colors[167] [169] The latter principle is also used alone, for example by means of pyrogallol[169] [170]

The phenol principle has also been applied in conjunction with inorganic chlorides[171]

All these methods are equally as accurate or less so than the Carr Price reaction None of these has been adopted for the determination of vitamin A by any official organization

*Carotene in the presence of vitamin A* can be determined by either chromatographic adsorption[17] or by colorimetric measurements[173] of the color developed by the Carr Price reaction (see above) or by direct spectroscopical examination

*The amount of free vitamin A in mixture with vitamin A esters* can be estimated by the behavior of the free vitamin toward methanol From an oily solution containing both the free and the esterified vitamin A, about 40–60% of the free vitamin, but no esters, is extracted with methanol[174] Molecular distillation is another method for the separation of the two forms[175]

## (b) Physical Methods

The extinction coefficient of the characteristic absorption band at 325–328 mμ can be used for the quantitative determination of vitamin A[176]

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[164] O Rosenheim and J C Drummond *Biochem J* 19 753 (1925)

[165] F H Carr and E A Price *Ibid* 20 497 (19 6)

[166] F Kobayashi and K Yamamoto *J Soc Chem Ind Japan* 27 1060 (19 4)

[167] A E Pacini and M H Taras *J Am Pharm Assoc* 26 721 (1937)

[168] N Bezssonoff *Bull soc chim biol* 11 294 (1929)

[169] W R Fearon *Biochem J* 19 888 (1925)

[170] T Moore *Lancet* II 219 (1929) S G Willimott and F Wokes *Ibid* II 8 (1927) O Rosenheim and T A Webster *Ibid* II 806 (1926) *Biochem J* 20 1342 (19 6)

[171] J Rosenthal and J Erdélyi *Biochem Z* 271 414 (1934)

[17] P Karrer and K Schöpp *Helv Chim Acta* 15 745 (193 )

[173] R Kuhn and H Brockmann *Z physiol Chem* 206 41 (1931)

[17] V Rits rt G P 636 ?27

[175] K C D Hickman *Ind Eng Chem* 29 968 1107 (1937)

[1] R A Morton *Practical Aspects of Absorption Spectrophotometry* Institute of Chemistry 1938 I M Heilbron and co workers *Biochem J* 22 987 (1928) 24 870 (1930) A Chevallier and P Chabre *Ibid* 27 298 (1933) K H Coward F J Dyer R A Morton and J H Gaddum *Ibid* 25 1102 (1931) A Chevallier and P Dubouloz *Bull soc chim biol* 18 190 (1936)

Care must be taken in choosing the solvent that is, either cyclohexane, ethyl or isopropyl alcohol has been recommended. The absorption bands vary somewhat both in position and in magnitude with change of solvents. As much as 10% increase (in ether) or 10% decrease (in chloroform) has been observed in comparison with the extinction coefficient of the same sample of vitamin A in alcohol.[177] It has been recommended[180] to check the instruments used against a suitable potassium chromate solution as a standard before and after each determination in order to increase the accuracy of the determinations. The factor for converting $E^{1\%}_{1\,cm}$ 328 mμ into International Units of vitamin A per gram is 1600, as accepted by the International Vitamin Conference 1934 ("conversion factor" = I. U./$E^{1\%}_{1\,cm}$).[178] The value for the conversion factor for pure vitamin A is believed to be about 2000–2100[179] or even 2375 but not enough experimental data are available to decide which value is correct.[18, 183] For vitamin A preparations from whale livers a conversion factor $E^{1\%}_{1\,cm}$ 325 mμ of 1200 has been recommended[184] due to the presence of considerable amounts of materials such as vitamin $A_3$.

A certain amount of the observed total absorption at 325–328 mμ may be due to carotenoids other than vitamin A and especially to glycerides. This is of importance when determinations of low potency materials are made whereas the error becomes negligible with preparations of high vitamin A potency.

As stated before vitamin A and the provitamins are destroyed by ultraviolet light. Therefore, the determination of the absorption band is somewhat inexact. At very low temperatures a differentiation between the band of vitamin A and the irradiation product of carotene is possible.[181] The selective decrease of absorption at 325 mμ produced by irradiating an alcoholic solution of a marine fish liver oil with mercury light of 365 mμ which destroys vitamin A, is said to be an accurate measure of the vita

[177] F. L. Smith, B. R. Stern and F. L. Young, *Nature* 141, 55 (1938). E. M. Hume and H. Chicks, *Med. Research Council Brit. Special Rept. Series* 1935, No. 202.

[178] See also E. M. Hume, *Nature* 139, 467 (1937).

[179] H. N. Holmes and R. E. Corbet, *J. Am. Chem. Soc.* 59, 2043 (1937). T. H. Mead, S. W. F. Underhill and K. H. Coward, *Biochem. J.* 33, 589 (1939).

[180] J. B. Wilkie, *J. Assoc. Official Agr. Chem.* 22, 465 (1939).

[181] F. P. Bowden, S. D. D. Morris and C. P. Snow, *Nature* 131, 58 (1933).

[182] This high factor could, for example, be caused by a hyperactivity of the U. S. Pharmacopoeia Reference Oil or by an inadequate utilization of carotene (less than 50%) when the U. S. Pharmacopoeia Reference Oil was initially standardized. It has also been debated if the values are not exaggerated due to deterioration of the Standard Reference Oil. J. R. Edisbury, *Analyst* 65, 484 (1940).

[183] J. G. Baxter and C. D. Robeson, *Science* 92, 202 (1940).

[184] J. R. Edisbury, *Analyst* 65, 484 (1940).

min[185] The effect on other constituents, particularly carotenoids is however, unpredictable

Fluorescence offers a possible method of differentiating between vitamins $A_1$ and $A_2$ Vitamin $A_1$ exhibits a characteristic green fluorescence, vitamin $A_2$ a reddish fluorescence[186] Upon irradiation both fluorescences fade

### (c) Biological Methods

The efficacy of vitamin A preparations is tested on young rats In the prophylactic method the substance with the unknown vitamin A content is added to a vitamin A free diet, and the growth of rats is compared with the growth rate of normally growing animals Another method uses young rats which have ceased growing due to a vitamin A deficiency but gain weight after the addition of vitamin A The results are compared with an International Standard preparation of β carotene, which, as has been pointed out before is converted under suitable conditions in the rat into an equimolecular quantity of vitamin A The latter method is recommended by the U S Pharmacopoeia

In addition the appearance of a cornified epithelium in the vaginas of spayed rats (colpokeratosis) on a vitamin free diet and its rapid disappearance following vitamin A feeding have been used for the determination of vitamin A

Another biological test which has been used for determining the physiological minimum requirement is the evaluation of the dark adaptation of the eye after exposure to bright light Individuals who have a partial or complete deficiency of this vitamin show some degree of night blindness[187]

## 21 Standards

The International Standard for vitamin A was adopted by the International Vitamin Conference 1934 and subsequently adopted for the United States Pharmacopoeia

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[185] A Chevallier Z Vitaminf sch 7 10 (1938) N K De Indian J Med Research 24 3 (1937) O Notewark Biochem J 29 12 7 (1935)

[186] R Greenberg and H Popper Proc Am Physi l Soc 1940 71

[187] See chapter on the visual purple This method was originally described by P C Jean and J Zentmire J Am Med Assoc 102 89 (1934) and has been modified by several workers

1 I U (International Unit) = I U S P Unit
= 0 6 $\gamma$[188] of pure $\beta$ carotene m p 184 optically in active dissolved in coconut oil[189] with addition of hydroquinone
= 1 5–2 Sherman Units

1 g of U S P cod liver oil must contain at least 850 U S P Units of vitamin A
1 g pure carotene contains 1 670 000 I U
1 g pure vitamin A contains 4 500 000 I U

1 C L O Unit (Cod Liver Oil Unit) = 125 $\gamma$ $\beta$-carotene
= 208 U S P Units
= 10 Lovibond Units
= 50 Lovibond Units (Wolff)
= 550 blue units (Moore)

1 blue unit (Moore) = Carr Price value 0 0182
= $E_{1\,cm}^{1\%}$ 328 m$\mu$ 0 000373
= 60 I U

The ratio of $E_{1\,cm}^{1\%}$ 620 m$\mu$ to $L_{1\,cm}^{1\%}$ 620 m$\mu$ ($L$—620 m$\mu$ value) is 1 30 ± 0 03

The ratio of the activity of $\beta$ carotene and of vitamin A as reported above has been determined under the specified conditions of the biological test An equality of an International Unit of vitamin A and of carotene can be claimed only under those conditions and is actually quite different for the metabolism of a normal growing organism To express the requirement of an organism, double standards must be recognized one for carotene and another one for vitamin A (see also page 95) (For a discussion of the various influences on vitamin A and on carotene utilization by the organism see page 84 )

At the level of a physiological optimum the ratio of efficiency of vitamin A to carotene by weight is about 6 1 and at a level that assures significant storage and successful reproduction about 10 1

It would be well to express the values of vitamin A potency not only in units but it should be required to indicate the method which was used for the determination for example U S Pharmacopoeia method, spectro scopic method etc

## 22 Physiology of Plants and Microorganisms[190]

Plants and also microorganisms contain only provitamins A The presence of vitamin A has never been demonstrated and there is ample

[188] 1 $\gamma$ = 0 001 mg = 10$^{-6}$ g

[189] The st ndard of 0 6 $\gamma$ $\beta$ c rotene as on Inte n t on l Un t repl ed th e l r tand rd set up in 1931 O e Stand rd Un t was then defined by ag eem nt to repr ent 1 n g of carotene m p 179 prepared acco d ng to the method of W ll tätter W1 n it beca ne evident that carotene s a mixture of varying amounts of som ric compound the b olog cal eq ival nt of the e rl er stand rd xpressed in weight of the pure $\beta$-carotene wa adopted as Internat onal Standa d

[190] See also O A Bes ey and S B Wolbach *J Am Med Assoc* 110 2007 (1937)

evidence that plants and many microorganisms do not need this vitamin Whatever the function of this vitamin may be in the animal organism, either plants do not need this function or they take care of it by some other means On the other hand, the relatively high concentrations of carotenoids and especially of provitamins in plants are striking These compounds generally appear in all growing parts of the plant and especially in the shoots It thus appears that provitamins A have a very definite purpose in the plant organism What this is, has however, not been elucidated It is suspected that plants need provitamins A for proper growth It has also been assumed that at least part of the action of these compounds is related to the efficient utilization of light by the plant organism Plants are definitely photosensitive and phototropic responses have been shown to be associated with the presence of carotenoids[191]

## 23 Animal Physiology

### (a) *Metabolism of Provitamins A and of Vitamins A*

Both provitamins A and vitamins A are preferably administered orally although they can be applied parenterally, for example, by subcutaneous or intramuscular injection They are also efficiently absorbed through the skin[192]

Provitamins A and vitamins A are absorbed from the intestinal tract Carotenes in colloidal water solutions are efficiently utilized, for example, by the rat[193] to prevent vitamin A deficiency symptoms Proper absorption of vitamin A is on the other hand related to the presence of fat The nature of the fat or oil, which is used for example, for dissolving the vitamin, determines to a certain extent the degree of utilization of the vitamin The more unsaturated the oil is, the better the absorption From mineral oils, vitamin A is absorbed only sparingly The absorption of provitamins A and of vitamins A is furthermore linked to the presence of bile acids and apparently also of pancreatic lipase In jaundice and in choledochocolonostomized dogs carotene is not absorbed[194] There is evidence that the absorption mechanism of birds is somewhat different in that these animals need some external supply of fat for proper utilization of

[191] E S Johnston *Smithsonian Inst Pub* 92 11 (1934) G Wald and H G du Buy *Science* 84 47 (1936)

[192] W v Drigalski *Z Vitaminforsch* 3 260 (1934) H J Huber and H Rocholl *Klin Wochschr* II 1143 1707 (1935)

[193] B N Majumdar *Ind J Med Research* 27 413 (1939)

[194] J D Greaves and C L A Schmidt *Am J Physiol* 111 492 502 (1935)

carotenes while vitamin A is apparently absorbed efficient[ly if] practically no fat is given in the diet[19]

The absorption of provitamins and of vitamins A from t[he] tract is a rapid process The absorption of vitamin A in rats reaches a maximum in three to five hours after administration, a[s] the disappearance from the intestinal tract and the rise of the [vitam]in A concentration in the blood[196] The rate of absorption of the provitamins A is somewhat slower and reaches its maximum at seven to eight hours after administration Vitamin A esters are hydrolyzed in the gut prior to absorption,[197] but it seems that the vitamin is re esterified during or soon after the absorption[198]

Both provitamins A and vitamins A are transported through the organism by the blood The carotenoids are present in the blood serum while the erythrocytes are devoid of these compounds They appear to be solubilized by the formation of loose protein addition compounds There seems to exist a minimum normal blood level of vitamin A and perhaps also of provitamin A This level is independent of the amount of stored vitamin or provitamin and is only temporarily increased during times of absorption from the intestines In vitamin A depleted rats for example, the vitamin A content of the blood plasma is zero During times of low vitamin A intake, the level in the blood is directly related to the amount fed After the concentration in the serum has reached what appears to be an optimal level that is about 100 International Units per 100 cc of plasma the concentration does not increase in the blood even when excessive amounts are given On the other hand no vitamin A is stored during low vitamin A intake while this vitamin is rapidly deposited in the liver[199] when optimal doses are administered

Provitamins A and vitamins A are removed from the blood by the reticulo endothelial system[200] Large amounts of vitamin A are stored in the liver There are no quantitative data available which would indicate what the minimum amount stored in the livers of any species or of man should be The vitamin is present in the liver mainly in the Kupffer and epithelial cells in the latter in lipoid droplets and diffusely in the cytoplasm Vitamin A is also stored to a certain extent in the adrenal cortex

[195] W C Russell M W Taylor H A Walker and L J Polskin *Proc Am Soc Biol Chem* 1941 CIX

[196] S W Clausen *J Am Med Assoc* 101 1384 (1933)

[197] B L Gray K Morgareidge and J D Cawley *Proc Am Soc Biol Chem* 1940 XXXVII

[198] J C. Drummond M F Bell and E T Palmer *Brit Med J* I 1208 (193 )

[199] J M Lewis O Bodansky K F Falk and G McGuire *Proc Soc Exptl Biol Med* 46 248 (1941)

[200] B Ahmad *Current Sci* 2 477 (1934) F Lasch and D Roller *Klin Wochschr* 15 1636 (1936)

in the form of small globules, in the *corpus luteum* and in the lutein cells of the ovary Generally, fat cells contain small amounts Traces may be found in the interstitium of the renal cortex and papilla, in the alveolar septum of the lungs and the intermediary part of the pituitary [201]

Vitamin A is stored in the body mainly in esterified form, although small but definite amounts of the free vitamin are always present The stored vitamin A esters are derivatives of a series of saturated and unsaturated fatty acids Whether or not certain specific acids are preferentially selected by the organism for esterification purposes is not known [202]

The ability of the animal organism to store large amounts of vitamin A has been discussed The rat, for example, may store in a few days enough vitamin A to supply its nutritional needs for several months [203] In the mammal this storage is very efficient after times of vitamin A depletion or of low vitamin A intake The efficiency of this storage decreases however, with an excess intake over the minimum physiological requirement [204] *On the other hand, the stored vitamin A is used rapidly when the food in* take is lacking in this vitamin until a certain apparently critical vitamin A level in the liver is reached Thereafter, the rate of depletion is much slower [205] These observations suggest that a special vitamin A utiliza tion mechanism exists in the organism Provitamins A are stored in small but definite amounts Examples have been given in the section on the occurrence of provitamins A (see page 38)

Vitamin A and to a certain but considerably smaller extent also provita *mins A are secreted into milk* In cow s milk, the ratio of vitamin A to provitamin A varies considerably with the species breed nutrition, etc but the total is of the same order of magnitude regardless of the breed [206] The vitamin A activity of the milk is fairly constant regardless of the time of the day when the milk is secreted but is to a certain extent influenced by the vitamin A potency of the food Human milk is about 5 to 10 times as rich in vitamin A potency as cow s milk The colostrum of all species investigated contains a much higher vitamin A potency than the milk In the case of humans the colostrum contains 2 to 3 times, and in the case of cows 10 to 100 times the amount present in milk [207] Both provitamins and vitamins A are also secreted in eggs A special mechanism apparently

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[201] H Popper and R Greenberg *Proc Am Physiol Soc* 1940 14C

[202] E L Gray K C D Hickman and F F Brown *J Nutrition* 19 39 (1940)

[203] A W Davies and T Moor *Biochem J* 31 172 (1937)

[204] L F Booher and M B Porter *Proc Am Soc Biol Chem* 1941 XCI

[205] A W Davies and T Moore *Biochem J* 29 147 (1935)

[206] C A Baumann H Steenbock W M Dee on and I W Rupel *J Biol Chem* 105 1167 (1934)

[207] W J Dann *Biochem J* 30 1644 (1936)

exists for the passage of vitamin A through the placental wall The vitamin A content of all newborn animals is low but a vitamin A deficiency has never been found when the mothers have had an ample supply of this vitamin After birth the vitamin A content rises rapidly and reaches the normal level of the adult within a few days or weeks

Both provitamins and vitamins A are in general readily metabolized No excretion takes place through the kidneys In the feces a certain proportion of the ingested provitamins may be found, especially when excessive amounts are fed or when the absorption mechanism is hampered

### (b) *Physiological Action of Provitamins A and of Vitamins A*

The physiology of man and of animals as influenced by vitamin A must ultimately be traced to the mechanism of the vitamin A action This mechanism is for the most part unknown

It has already been discussed that provitamins A are absorbed in the intestinal tract and to a certain extent stored in the animal organism It has also been shown that provitamins A are converted in the organism into vitamin A Except as precursor of vitamin A carotenes as such are not known to be active in the animal economy The possibility should however, not be overlooked that provitamins may act in a specific way of their own Thus it may be significant that provitamins A are stored in man and in animals in a number of special organs primarily in the glands which are concerned with the functions of reproduction In invertebrata which are able to synthesize their own specific carotenoids[208, 209] these carotenoids apparently play a definite and important role in metabolic processes It has also been suggested that for example in mussels carotenoids may play some role in gametogenesis[209] In this connection it is well to recall the action of various carotenoids of algae for which these compounds are secretion products concerned with the development of motility and with conjugation of the gametes[210] While these carotenoids in lower animals are not necessarily provitamins A, they are closely related to them Thus, it seems possible that the provitamins A have an influence upon the maintenance of a normal mechanism of the sex apparatus It has furthermore been shown repeatedly that the sex organs of rats kept on a vitamin A depleted diet degenerate This may of course be explained as meaning that either vitamin A or provitamins A are necessary It is however also possible that the sex glands do not utilize the provitamins A proper but

[208] E Lederer *Bull soc chim biol* 20 554 567 611 (1938)
[209] B T Scheer *J Biol Chem* 136 75 (1940)
[210] F Moewus *Naturwissenschaften* 27 97 (1939)

have a mechanism of their own for converting the provitamins into active compounds such as, for example vitamin A

Another unsettled question of physiological importance is the problem of whether or not the vitamin A itself acts in the organism It has previously been stated that vitamin A is stored in the liver mainly in the esterified form, but that some free vitamin A can always be found Thus it is possible that the vitamin is esterified only for the purpose of storage and it is probable that the vitamin acts in the free form This question cannot be decided definitely until the mechanism of the vitamin A action has been elucidated

A possible clue to this mechanism may be seen in the increase of purines in the growing vitamin A depleted tissue after this vitamin has been administered [11] Purines are necessary building units of cell nuclei Actually all primary and secondary symptoms of a vitamin A deficiency can be explained on this basis They all constitute cellular changes in the most sensitive parts of the body, such as in the respiratory mucosa, the salivary glands, the intestinal tract and finally in the skin in general Thus, the principal role of vitamin A is to stimulate the building of cells

Beyond this fundamental and specific action of vitamin A a general influence on the basic metabolism has also been considered A possible connection with the oxidation mechanism has been suggested and was apparently supported by experiments which showed that the oxygen consumption of livers in the presence of iron containing porphyrines increases with the amount of vitamin A present [21] This theory has not however, been further advanced and appears to be somewhat doubtful and unspecific in view of the fact that it has been possible to connect some of the other vitamins with definite stages of the oxidation reduction mechanism of the living tissue This has however, not been possible in the case of vitamin A

Vitamin A seems to exert however, some non specific functions upon the fat and carbohydrate metabolism The necessity of the presence of fat has been discussed in connection with the absorption and storage of this vitamin) Furthermore during avitaminosis the amount of fat deposited in the organism decreases slowly but the fat deposits are restored upon administration of vitamin A Similarly the cholesterol content in the organism decreases during times of low vitamin A intake On the other hand, excess doses of vitamin A cause an appreciable increase of the cholesterol content in blood and in the brain Similar relations have been

[211] H v Euler and G Schmidt *Z physiol Chem* 2 3 215 (1934)
[212] H v Euler and L Ahlström *Ibid* 204 168 (1932)

found for the carbohydrate metabolism for example for the glycogen content of the liver

Vitamin A acts, however, in the body also in some other specific way In conjunction with a specific protein vitamin A plays an essential role in the visual purple This will be discussed in a special section (see below) Whether or not vitamin A acts in conjunction with other proteins in some other specific functions is not known It has been found experimentally that in vitamin A deficiency a marked decrease in the concentration of blood serum esterase an appreciable decrease in hepatic esterase and a marked increase in hepatic lipase occur [13] These findings need confirmation and should not necessarily be interpreted as indicating a relationship of vitamin A to specific enzyme systems

Vitamin A is mobilized in the organism in a very special way whenever a state of disease occurs Thus the intake of ethyl alcohol by dogs[214] or by human beings causes a specific mobilization of the vitamin A stored in tissues since both the blood level[215] and the dark adaptation test[216] indicate higher vitamin A concentrations than normal In many diseases such as fever etc an increased demand for vitamin A has been demonstrated [217] The turnover of vitamin A in the liver can also be effected by injection of carcinogenic compounds, such as dibenzanthrene benzopyrene and methyl cholanthrene [218] This effect is not necessarily specific for vitamin A although it appears to be

## (c) The Visual Purple

The energy of dim light, which strikes the eye is transformed into nerve impulses by the visual purple on the outside end of the retina Color and light of high intensity are perceived by the cones of the retina

Rhodopsin the visual purple bleaches out under the influence of light and regenerates in the absence of light Rhodopsin is a carotenoid albumin with an absorption maximum at 500 mμ The prosthetic group retinene, is a carotenoid of unknown composition [19] but related to a form

[213] B Sure M C Kik and K S Buchanan Proc Soc Exptl Biol Med 35 209 (1936)

[214] S W Clausen W S Baum A B McCoord J O Ryden and B B Breese Science 92 318 (1940)

[215] S W Clausen B B Breese W S Baum A B McCoord and J O Ryden Ibid 93 21 (1941)

[216] L B Pett Ibid 92 63 (1940)

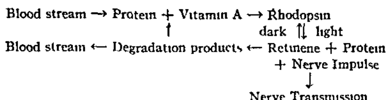
S W Clausen J Am Med Assoc 111 144 (19 S)

[218] C A Baumann and F G Foster Proc Am Soc Biol Chem 1941 XII

[219] L S Fridericia and E Holm Am J Physiol 73 63 (1925) E Holm Ibid 73 79 (1925) S Hecht A M Chase S Shlaer and C Haig Science 84 331 (1936) S Hecht Physiol Rev 17 239 (1937) S Hecht A M Chase and S Shlaer Science 85 567 (1937) C Haig S Hecht and A J Patek Ibid 86 534 (1937) S Hecht and J Mandelbaum Ibid 88 219 (1938) S Hecht and J

(Footnote continued on page 90)

of carotene Under the influence of light the red color disappears and an unstable orange compound transient orange, is formed This changes probably in a dark process, to indicator yellow which appears to be a loose protein compound of retinene Retinene can be extracted from the visual yellow with chloroform, is ligroin soluble and has an absorption maximum at 385 mμ in chloroform ($SbCl_3$ color reaction yields a maximum at 664 mμ) Retinene can also be obtained from rhodopsin through acid or alkali hydrolysis The visual yellow breaks down further to vitamin A and a specific protein In the dark, a partial resynthesis of rhodopsin occurs The pigmented layers of the eye contain large amounts of vitamin A, which is used for the synthesis of rhodopsin Vitamin A deficiency therefore causes as one of its first symptoms a decreased ability for dark adaptation since not enough vitamin A is available for the synthesis of rhodopsin




Vitamin A is replaced in fresh water fish by vitamin A which replaces also the vitamin A in the visual purple and is called porphyropsin (absorption maximum at 525 mμ) [220] In some marine fish compounds of different absorption characteristics are found which indicate the presence of additional factors A (or mixtures of rhodopsin and porphyropsin) [221]

### (d) *Relation of Vitamins A to Other Vitamins, Hormones, etc*

In the general chapter on the interrelationship between the vitamins and the hormones it has been pointed out that the physiological equilibrium of any organism is disturbed if one of the vitamins is not supplied regularly and adequately

Of special interest in the case of vitamin A are the claims concerning an antagonism with the other fat soluble vitamins especially vitamin D and the antagonism with some of the water soluble vitamins especially vitamin C It has been stated that a vitamin A deficiency causes similar

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[219] *(Continued)* Mandelbaum *Am J Physiol* 130 651 (1940) G Wald *J Gen Physiol* 18 905 (1935) G Wald and A B Clark *Am J Physiol* 116 157 (1936) G Wald *J Gen Physiol* 19 351 781 (1935) 20 45 (1936) *Nature* 139 587 (1937) R J Lythgoe *J Physiol* 89 331 (1937)

[220] G Wald *Nature* 139 1017 (1937)

[221] G Wald *Ibid* 139 1017 (1937) R J Lythgoe and K Tansley *Proc Roy Soc (London)* B120 95 (1936)

disturbances as an excess supply of vitamin D The vitamins A and D however do not exert any synergistic or antagonistic action at the normal optimal concentrations The natural combination of vitamins A and D in liver oils prevents any signs of hypervitaminosis even if given in large amounts due to the alleged antagonism of these vitamins which in this case even represents a synergism Antagonism of vitamins A and C is seen when both vitamins are given in excess at the same time since no vitamin A hypervitaminosis develops [2 3]

There is apparently a relationship between the vitamin A metabolism and vitamin E During vitamin E deficiency in the rat no vitamin A is stored in the liver and the amount of vitamin reserves in the liver is markedly reduced even when ample quantities of vitamin A are administered [22,4] Thus an acute secondary deficiency of vitamin A develops Whether this is due only to the non specific antioxidant power of vitamin E compounds or to a specific physiological effect is not known

Among the relations of vitamin A to hormones the alleged antagonism to the hormone of the thyroid gland thyroxine has been extensively studied [224] Toxic effects of excesses of this hormone are said to be overcome by the administration of large amounts of vitamin A Rats given thyroxine were depleted of vitamin A much more rapidly than normal rats [22] No evidence for a specific antagonism could be obtained, however [26] but a temporary disturbance in the metabolic rate has been found upon administration of large doses of vitamin A to normal and thyroidectomized rats [2]

The secretion of the hormones of the anterior lobe of the pituitary gland seems to be related to the vitamin A metabolism since in frogs the glycogenotropic hormone could be detected in the liver only after intake of vitamin A[2 8] together with vitamin E

## 24 Avitaminosis and Hypovitaminosis

The action of vitamin A can be regarded essentially as a stimulus to the building of new cells Thus vitamin A deficiency causes retarded growth This symptom is however not specific for a vitamin A deficiency

Specifically, lack of vitamin A causes atrophy of the epithelium with substitution of a stratified keratinized epithelium for the normal epithelial

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[111] A Scheunert Naturwissenschaften 28 297 (1940)

[111] H W ndt and H Schroeder Z Vitamin forsch 4 206 (1933)

[111] T Moore *Biochem J* 34 1321 (1940) A W Davies and T Moore *Natu* 147 794 (1941) H v Euler and E Klussmann *Z physiol Chem* 213 21 (1932)

[1] J D Greaves and C L A Schmidt *Am J Physiol* 116 456 (1936)

[1m] C H Baum n and T Moore *Biochem J* 33 1639 (1939)

[11] R F Sheets a d H C Struck *Proc Am Physiol Soc* 1941 2 u

[1m] L Képnov *Compt rend* 209 358 (1939) 210 188 (1940)

structure[229] This is first observed in the respiratory mucosa The mucosa in the mouth and the salivary glands is next affected causing in turn a greater susceptibility to infections Finally the epithelial mucosa of the eyes, the intestinal tract the urethra, the kidney etc is degenerated The epithelium of the vagina is especially sensitive to vitamin A deficiency causing the appearance of colpokeratosis and of the so called senile vaginitis[230] In man a general dryness of the skin and a hyperkeratosis of the hair follicles are observed The hair becomes dry and lusterless Dermatosis a form of keratosis or cutaneous eruption, occurs on the fore arms and thighs and later practically all over the body[231] Gastro intestinal disorders may occur[232] and stones may be formed in the bladder and in the kidney (urinary calculi)[233] In some experimental animals for example, in pigs and in rats certain neurological lesions also were ob served[234]

The various eye lesions caused by vitamin A deficiency are especially significant *An early symptom of a hypovitaminosis is often the so called* night blindness (nyctalopia or functional hemeralopia)[235] At later stages of the deficiency a softening of the cornea, followed by perforation (kerato malacia) and a dry, lusterless condition with white deposits on the scleral conjunctiva (nutritional xerophthalmia) occur Vitamin A deficiency may also contribute to the development of myopia[236] In pigs from sows kept on a vitamin A depleted diet during pregnancy total blindness was observed and some animals had no eyes at all[237] Lack of vitamin A may cause blindness by a constriction of the optic nerve associated with a stenosis of the optic canal[238]

During times of vitamin A deficiency, the normal functioning of the re productive system is hampered In the female rat, the vaginal mucous membrane becomes cornified, as previously stated There is also some adverse influence on the ovary, and normal reproduction does not occur

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229 S B Wolbach and P R Howe *J Exptl Med* 42 [illegible] (19[illegible])

230 J W Simpson and K E Mason *Am J Obstet Gynecol* 32 12[illegible] (1936)

231 L J A Löwenthal *Arch Dermat Syphilol* 28 700 (1933) C N Frazer and C K Hu *Ibid* 33 825 (1936) J B Youmans and N B Corlette *Am J Med Sci* 195 644 (1938)

232 R Roller *Z Klin Med* 130 163 (1936) G Will *Klin Wochschr* 15 1281 (1936)

233 W M Kerns *Wisconsin Med J* 36 170 (1937) C C Higgins *J Urol* 36 168 (193[illegible]) Su[illegible] *Gynecol Obstet* 63 23 (1936) M Meltzer *N Y State J Med* 37 865 (1937)

234 S B Wolbach and O A Bessey *Science* 91 599 (1940)

235 I O Park *J Oklahoma Med Assoc* 28 307 (1935) 29 123 (1936) P C Jeans and Z Zentmire *J Am Med Assoc* 106 996 (1936) P C Jeans E Blanchard and Z Zentmire *Ibid* 108 451 (1937) H Frandsen *Acta Ophthalmol* (Supplements) 4 (1935) H Jeghers *New Engl J Med* 216 51 (1937) *Ann Internal Med* 10 1304 (1937)

236 H Miller *Am J Opthalmol* 23 296 (1940)

237 J L Novaes *Hora Medica* 11 84 (1939)

238 L. A. Moore *J Nutrition* 17 443 (1939)

In the male rat, the testes degenerate in severe cases of A avitaminosis

Vitamin A deficiency during the period of tooth development can impair tooth structure by causing an atrophy and metaplasia of the enamel organ [239] Thus a hypoplastic tooth with thin defective enamel is formed The rate of apposition of dentine is altered in vitamin A deficiency while the life span of the formative cells is not affected [40] In rats the incision teeth lose the deep orange pigment in the enamel which is restored upon administration of vitamin A [241]

An early symptom of vitamin A deficiency is the decrease of the normal vitamin A and carotenoid level in blood The normal level for children, when determined under specified conditions is from 5 5 to 27 3 units of vitamin A per cc of blood and the carotene level is from 3 1 to 75 units per cc A vitamin A level of less than 3 units of vitamin A indicates avitaminosis and a value of less than 3 units of carotenes points to a state of hypovitaminosis [242] During avitaminosis vascular lesions in practically the entire arterial system have also been observed [243]

The state of hypovitaminosis or avitaminosis may be caused besides by an insufficient intake of vitamin A also by an impaired intestinal absorption as has been found in cases of congenital obliteration of the bile ducts, fibrosis of the pancreas celiac disease and others [24]

## (a) *Clinical Test Methods*

1 **The Dark Adaptation Test** Night blindness in the absence of eye disease and of a hereditary tendency in that direction may often be a manifestation of vitamin A deficiency [244] For actual diagnosis a number of different adaptometers [24] which are modified photometers are available on the market The test is carried out by one of two methods The patient after a preliminary period in the dark is exposed to subdued light and the minimum amount of light that is visible is measured This method has been found especially useful for the determination of a vitamin A deficiency in infants [246] In the other method the visual purple in the

[239] S B Wolbach and P P Howe *J Exptl Med* 42 753 (1925)

[240] L Schour M C Smith and M M Hoffman *Proc Soc Exptl Biol Med* 39 447 (1938)

[241] J T Irving and M B Richards *Nature* 144 908 (1939)

[242] C D May K D Blackfan J F McCreary and F H Allen *Am J Diseases Children* 59 1167 (1940)

[243] L Opper *Proc Soc Exptl Biol Med* 40 449 (1939)

[244] J B Feldman *Arch Ophthalmol* 12 81 (1934) 15 1004 (1936) 17 648 (1937) 18 821 (1937)

[245] G S Derby P A Chandler and L L Sloan *Arch Ophthalmol* 3 31 (1930) S Hecht and J Mandelbaum *J Am Med Assoc* 112 1910 (1939) S Hecht and S Shlaer *J Optical Soc Am* 28, 269 (1938)

[246] C Friderichsen and C Edmund *Am J Diseases Children* 53 89 1179 (1937)

retina is bleached by means of bright light, and the minimum time necessary to recover clear vision is then determined [247]

2 **Determination of the Vitamin A Content of Blood** A number of different methods have been developed for the determination of the vitamin A content of blood Assays can be made with the serum alone since the blood corpuscles do not contain significant amounts of this vitamin The actual determination can be carried out spectroscopically in alcohol solution after the blood has been treated with sodium sulfate [248] It has also been recommended to test for vitamin A in blood by determining the amount of material destroyed by ultraviolet light [249] For this purpose the blood is hydrolyzed and the non saponifiable fraction is determined spectroscopically for its apparent vitamin A content Irradiation with monochromatic light of 365 mμ causes specific changes of the absorption maximum of vitamin A from which the actual amount of vitamin A present in the solution can be calculated This method cannot be used, however, if appreciable amounts of carotene are present The best method for determining both vitamin A and provitamins A in blood consists in a combination of a spectroscopical determination with the Carr Price color reaction [250] For this purpose, blood serum is extracted with a mixture of ethanol and petroleum ether [251] The petroleum ether extract is used for a spectroscopical determination of the carotenoids[252] since bile pigments are not extracted under the specified conditions For practical purposes the total carotenoid value is considered to consist of a 50-50 mixture of β carotene with the inactive xanthophyll The vitamin A content of the extract is then determined according to the Carr Price technic (see page 78) The vitamin A content of blood can of course also be determined by the biological methods described for the determination of vitamin A

3 **Demonstration of Keratinized Epithelial Cells** In this test the presence of cornified cells in scrapings from the cornea nose and mouth and in secretions from the trachea bronchi kidneys and vagina is investigated by special staining methods which give the keratinized cells definite colors while leaving normal cells colorless Several reagents have been suggested, among which is 1% methylene blue in a 3% acetic

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[247] L B Pett *J Lab Clin Med* 25 14[illegible] (193[illegible])

[248] A Chevallier and Y Choron *C [illegible] rend [illegible]* 118 883 ([illegible])

[249] A Chevallier Y Choron and R Matheron *Ibid* 127 [illegible]41 (1938)

[250] C D May K D Blackfan J F McCreary and F H Allen *Am J Diseases Children* 59 11[illegible] (1940)

[251] S W Clausen and A B McCoord *J [illegible]* 13 [illegible]3 (1938)

[252] W S Ferguson *Analyst* 60 680 (193[illegible])

acid water solution (Mallory's stain for diphtheria bacilli) which dyes the pathological cells deep red[253]

4 Vitamin A Absorption Test The efficiency of the intestinal absorption of vitamin A is studied in this test[254] A standardized test dose of vitamin A is given by mouth and the vitamin level of the patient's blood is assayed before and at definite intervals after the vitamin intake An average rise of at least 50 to 130 units of vitamin A per cc should be observed in from three to five hours otherwise a subnormal efficiency of the intestinal absorption is indicated

## 25 Hypervitaminosis

No indications are known for any toxic effects of excessive doses of provitamins A It can be assumed that a regulatory mechanism in the body takes care of handling amounts of provitamin A which are in excess of the animal's actual needs

The toxicity of pure crystalline vitamin A has not been investigated so far Using vitamin A concentrates doses in excess of 100,000 units per day are harmful for rats[255,256] The pathological changes observed include retarded growth, hemorrhages, especially in the mucous membranes, and abnormal rarefaction and fragility of the bones

## 26 Vitamin A Requirements

All mammals birds and fish which have been investigated, utilize vitamin A This does not mean however that all animals need this factor Thus it has been demonstrated[257] that the cockroach (*Blatella germanica* L.) needs no provitamin or vitamin A in the diet and is able to function normally throughout life without this vitamin It is therefore suspected that other animals such as certain insects do not require vitamin A

Double requirement standards must be recognized, one for vitamin A and one for carotene (provitamin A) since the International Units of carotene and of vitamin A are equal only under the specified conditions of the biological test but not in the metabolism of a normal growing organism[258]

The vitamin A requirements of adult mammals can be correlated to the body weight Fairly uniform requirements have been established for

[253] K D Blackfan and S B Wolbach *J Pediat* 3 6?6 (1933)
[254] J Chesney and A B McCoord *Proc Soc Exptl Biol Med* 31 887 (1934)
[255] F B Vedder and C Rosenberg *J Nutrition* 16 57 (1938)
[256] T Ikegaki *Z Vitamin f sch* 7 113 (1938)
[257] R L Bowers and C M McCay *Science* 92 291 (1940)
[258] H R Guilbert C F Howell and G H Hart *J Nutrition* 19 91 (1940)

man [259] horse,[258] dog, [260] cattle,[258, 61] sheep,[258, 261] swine,[258 261 262] rabbit,[263] rat [64] and hedgehog [265] All show approximately the same requirements, namely, 25 γ of β carotene (corresponding to 40 International Units) or 4 γ (20 International Units) of vitamin A per kilogram of body weight [258, 261 266] These values represent the minimum for normal growth without showing any clinical symptoms of vitamin A deficiency, but little or no storage of the vitamin occurs at these levels About three times the minimum amount of vitamin A (12 γ or 60 International Units) and five times the minimum amount of β-carotene (125 γ or 200 International Units) is considered minimum for significant storage and reproduction The optimum vitamin A requirement for an average adult is then about 5000 International Units of vitamin A or 15 000 International Units of carotene Increased amounts are recommended for pregnant and nursing women and during adolescence [67] The allowances as recommended by the Food and Nutrition Board of the National Research Council are reprinted on page 613

The vitamin A requirements of animals other than mammals are less well known Birds apparently need vitamin A in an amount of the same order of magnitude as mammals Growing chicks need about 95 to 125 γ of carotene per day [68] (or about 1800 International Units of vitamin A per pound of feed) but for laying stock 200–500 γ of carotene have been recommended [269] (This amount corresponds to a diet of yellow corn or to an addition of 2 5–5% of a good grade alfalfa meal )

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259 See the discussion by L E Booher *J Am Med Assoc* 110 1920 (1938)

260 P D Crimm and D M Short *Am J Physiol* 118 477 (1937)

261 H R Guilbert and G E Hart *J Nutrition* 10 409 (1935) H R Guilbert R F Miller and F H Hughes *Ibid* 13 543 (1937)

262 H Mollguard *Biedermanns Zentr* (B Tierernähr ) 10 214 (1938)

263 P H Phillips and G Bohstedt *J Nutrition* 15 309 (1938)

264 R Kuhn and H Brockmann *Klin Wochschr* 12 972 (1933)

265 P Swomalainen *Skand Arch Physiol* 83 104 (1939)

266 J T Irving and M B Richards *Nature* 144 908 (1939)

267 Technical Commission for the Study of Nutrition Health Organization of the League of Nations *Geneva Bulletin* 7 April 1939

268 R M Sherwood and G S Fraps *Texas Agr Exptl Station Bull* M528 Sept 1936

269 J K Williams C E Lampman and D W Bolin *Poultry Sci* 18 268 (1939)

# VITAMIN $B_1$—
# THIAMIN

# VITAMIN $B_1$—THIAMIN[1]

## 1 Nomenclature and Survey

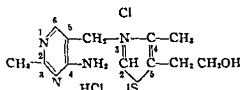
**Names**

Thiamin American nomenclature [2]
Aneurin European nomenclature [3]
Oryzanin Japanese nomenclature [4]
Torulin Historical name [5]
Polyneuramin Historical name [6]
Vitamin F Abandoned term [7]
The antineuritic anti beriberi vitamin

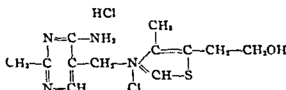
**Chemical name**

4 Methyl 5 β hydroxy ethyl *N* {[2 methyl 4 amino pyrimidyl (5)] methyl} thiazolium chloride hydrochloride

**Structure** *




Some authors prefer to write the formula of vitamin $B_1$ as follows




No agreement exists as to the manner of writing the pyrimidine nucleus except in purine derivatives The preferred form is the hexagonal to which the eye is accustomed and which best illustrates the conversion of vitamin $B_1$ to thiochrome

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[1] R R Williams and T D Spies *Vitamin B* New York 1938
[2] Council on Pharmacy and Chemistry *J Am Med Assoc* 109 952 (1937)
B C P Jansen *Nature* 135 267 (1935)
U Suzuki T Shimamura and S Odake *Biochem Z* 43 89 (191 )
C S Ide W H Evans B Moore C C E Simpson and A Webster *Biochem J* 6 234 (1912)
R I Jou *Science* 68 480 (1928)
[7] H C Sherman and J H Axtmayer *J Biol Chem* 75 207 (1927)

# VITAMIN $B_1$—THIAMIN[1]

## 1 Nomenclature and Survey

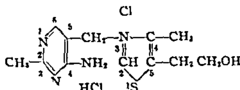
**Names**

Thiamin American nomenclature[2]
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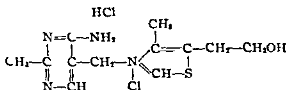
**Chemical name**

4 Methyl 5 β hydroxy ethyl *N* {[2 methyl 4 amino-pyrimidyl (5)] methyl} thiazolium-chloride hydrochloride

**Structure**[8]




Some authors prefer to write the formula of vitamin $B_1$ as follows




No agreement exists as to the manner of writing the pyrimidine nucleus except in purine derivatives. The preferred form is the hexagonal to which the eye is accustomed and which best illustrates the conversion of vitamin $B_1$ to thiochrome

[1] R R Williams and T D Spies *Vitamin B₁* New York 1938
[2] Council on Pharmacy and Chemistry *J Am Med Assoc* 109 95 (1937)
[3] B C P Jansen *Nature* 135 267 (1935)
[4] U Suzuki T Shimamura and S Odake *Biochem Z* 43 89 (191 )
[5] C S Ed W H Evans B Moore G C E Simpson and A Webster *Biochem J* 6 234 (1912)
[6] R I Jon *Science* 68 480 (19 8)
[7] H C Sherman and J H Axtmayer *J Biol Chem* 75 ?07 (19 7)

**Empirical Formula**



$C_{12}H_{17}ON_4SCl\ HCl$

**Efficacy**

1 g = 333 000 International Units

## 2 Chronology

1885 TAKAKI[8] prevented the occurrence of beriberi in the Japanese navy by changing the dietary ration

1893–1897 EIJKMAN[9] produced experimental polyneuritis (beriberi) in fowls by a diet consisting exclusively of polished rice and prevented the disease by dietary means

1912 FUNK[10] cured rats with dietary polyneuritis by the administration of water extracts of rice bran

1926 JANSEN and DONATH[11] isolated the vitamin in crystalline form from rice bran

1931 WINDAUS TSCHESCHE RUHKOPF LAQUER and SCHULTZ[12] isolated the *pure* vitamin $B_1$ from yeast and established its empirical formula

1936 WILLIAMS and independently GREWE elucidated the chemical structure of vitamin $B_1$ In the same year the synthesis of this vitamin was accomplished by WILLIAMS and CLINE and by ANDERSAG and WESTPHAL

1937 LOHMANN and SCHUSTER isolated crystalline cocarboxylase from yeast and proved its constitution as the pyrophosphoric ester of vitamin $B_1$

## 3 Occurrence

Vitamin $B_1$ is present in many plants Vegetables fruits and nuts contain small amounts, ripe peas and beans are rich sources but vitamin $B_1$ is found most prevalently in outside bran coats of grains (rice) and in yeast (baker s yeast and brewer s yeast) Generally speaking vitamin $B_1$ is present in high concentration in seeds that is in the nutritive material for the plant germ (see page 133) In most leaves the concentration of vitamin $B_1$ amounts to a constant value of about 25 International Units per 100 g , regardless of the botanical family [13]

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[8] K Takaki *Sei i Kai Med J* August 1885 April 1886 6 73 (1887) *Lancet* 1906 I 1369 1451 1520

[9] C Eijkman *Arch path Anat (Virchow s)* 148 523 (1897)

[10] C Funk *J Physiol* 45 75 (1912)

[11] B C P Jansen and W F Donath *Mededeel Dienst Volksgezondheid Nederland Indië* 1926 (Pt I) 186 *Chem Weekblad* 23 201 (1926) *Koninkl Akad Wetenschappen Amsterdam Wisk Natu k Afd* 35 923 (1926)

[12] A Windaus R Tschesche H Ruhkopf F Laquer and F Schultz *J physiol Chem* 204 123 (1932) *Nachr Ges Wiss Göttingen Math physik Klasse* 1932 207 342

[13] M Pyke *Biochem J* 34 330 (1940)

Some microorganisms are able to synthesize vitamin $B_1$. For example, certain bacteria exist in the intestinal tract of rats[14] (especially in the colon) and of nursing children (especially in the great gut) that synthesize vitamin $B_1$ which is then found in the feces. Bacteria which produce vitamin $B_1$ also live in the rumen of cattle, sheep, etc.[15]

Vitamin $B_1$ is widely distributed in the animal organism in different organs (liver, kidney) and in muscles (especially in the heart).[16] The actual amount is very small and varies greatly in different species. Pork muscles, for example, contain about eight times as much vitamin $B_1$ as beef muscles.[17] Storage of large amounts does not occur in the animal organism.

Vitamin $B_1$ occurs in nature as the free compound or in the form of its salts, as vitamin $B_1$ protein complex,[18] as vitamin $B_1$ pyrophosphoric acid ester (cocarboxylase), and as vitamin $B_1$ phosphorus protein complex. It is suspected that other vitamin $B_1$ containing compounds, for example, the monophosphate and other esters exist in tissues. The relative amounts of these forms vary considerably in different sources. Milk contains predominantly the free vitamin and the vitamin protein complex.[19] The first colostrum contains practically no protein complexes. In skeletal and in heart muscle the amount of the free vitamin is greater than the phosphorylated compound, whereas in brain and liver the cocarboxylase (and its protein complex) occurs predominantly.[20]

## 4. Isolation

Since vitamin $B_1$ is water soluble it is extracted, for example, from rice polish or yeast with cold water which is brought to pH 4.5 by the addition of mineral acid. From the aqueous solution the vitamin is adsorbed on activated fuller's earth or charcoal at pH 6.5.[21] Riboflavin, which is also present in the water extract, is not adsorbed under these conditions.[22] The vitamin is then extracted from the charcoal with dilute acids or from the fuller's earth with dilute alkali. By these methods, however, a con

[14] *J Hyg* 27 70 (19 7)

[15] S I Bechdel, H E Honeywell, R A Dutcher and M H Knutsen, *J Biol Chem* 80 231 (1928)

[16] H G K Westenbrink, *Arch néerland physiol* 17 560 (193 ), 19 116 (1932)

[17] C A Elvehjem, W C Sherman and A Arnold, *J Biol Chem* 109 XXIX (1935); R Hoagland, *J Agr Research* 83 431 (1929)

[18] J Houston and S K Kon, *Nature* 143 558 (1939)

[19] J Houston, S K Kon and S Y Thompson, *J Soc Chem Ind* 58 651 (1939); J Houston and S K Kon, *Nature* 143 558 (1939)

[20] S Ochoa and R A Peters, *Biochem J* 32 1501 (1938)

[21] H W Kinnersley, J R O'Brien and R A Peters, *Ibid* 27 232 (1933)

[22] S. Ohdake, *J Agr Chem Soc Japan* 10 400 (1934); R R Williams, R E Waterman and J C Keresztesy, *J Am Chem Soc* 56 1187 (1934)

siderable amount of vitamin is lost If, however, quinine salt solutions or salts of other organic bases are used, about five times as much vitamin is obtained[22] (approximately 90% of the vitamin $B_1$ originally present)

Impurities can be precipitated by barium hydroxide [23] benzoyl chloride in the presence of an excess of sodium bicarbonate [23] silver nitrate in acid solution[24] and by many other reagents The vitamin itself is precipitated by silver nitrate at pH 7 5 [23, 24] phosphotungstic acid at pH 4 5–5 5 [24, 25, 26] silicotungstic acid[27, 28] benzene sulfo chloride [29] picrolonic acid [30, 31, 32] Rufian acid [30] Reinecke acid [30] gold chloride in aqueous solution [24, 30, 33, 34] platinum chloride in alcoholic solution [24, 30, 35] and mercuric chloride in the presence of sodium acetate or carbonate [30] (but not by mercuric sulfate in acid solution [36]) etc

After the elution of the vitamin from the adsorbent, a combination of different precipitations is carried out The vitamin $B_1$ is thus obtained as the hydrochloride Four hundred and fifty pounds of rice polish or 2000 lbs of yeast yield about 1 g of the vitamin For practical isolation procedures see page 118

Vitamin $B_1$ and riboflavin, which usually occur together, are separated by precipitation of the riboflavin from an aqueous neutral or slightly alkaline solution with lead acetate, whereby vitamin $B_1$ remains in solution [35, 37, 38] Another method is a fractional adsorption first on charcoal at pH 4–5, which adsorbs all the riboflavin, and then on fuller s earth which adsorbs all the vitamin $B_1$ [39] On the other hand, vitamin $B_1$ may first be adsorbed from solutions of pH 3 on silica gel[40] or fuller s earth [41] The riboflavin remains in solution and can be recovered from the filtrate

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[23] A Seidell *J Biol Chem* 82 633 (1929)

[24] B C P Jansen and W F Donath *Mededeel Dienst Volksgezondheid Nederland Indië* 1926 (Pt I) 186 *Chem Weekblad* 23 201 (1926) *Koninkl Akad Wetenschappen Amsterdam Wisk Natuurk Afd* 35 923 (1926)

[25] H W Kinnersley J R O Brien and R A Peters *Biochem J* 27 ?3 (1933)

[26] H W Kinnersley and R A Peters *Ibid* 24 1856 (1930)

[27] B C P Jansen J P Wibaut P J Hubers and P W Wiardi *Rec trav chim* 52 366 (1933)

[28] B C P Jansen *Ibid* 48 984 (1929)

[29] A C van Veen *Z physiol Chem* 208 125 (1932)

[30] A Windaus R Tschesche H Ruhkopf F Laquer and F Schultz *J physiol Chem* 204 123 (1932) *Nachr Ges Wiss Göttingen Math physik Klasse* 1932 342

[31] S Ohdake *Proc Imp Acad (Tokyo)* 7 102 (1931) 8 179 (1932) 10 95 (1934)

[32] A Seidell and M I Smith *J Am Chem Soc* 55 3350 (1933)

[33] B C Guha *Biochem J* 25 931 (1931)

[34] B C Guha and J C Drummond *Ibid* 23 880 (1929)

[35] B C P Jansen J P Wibaut P J Hubers and P W Wiardi *Rec trav chim* 52 366 (1933)

[36] B C P Jansen H W Kinnersley R A Peters and V Reader *Biochem J* 24 1824 (1930)

[37] H Chick and M H Roscoe *Ibid* 23 504 (1929)

[38] J I Rosedale *Ibid* 21 1266 (1927)

[39] R D Greene and A Black *J Am Chem Soc* 59 1395 (1935)

[40] P A Levene *J Biol Chem* 79 465 (1928) *Science* 71 668 (1930)

[41] W D Salmon N B Guerrant and I M Hays *J Biol Chem* 80 91 (19 8)

## 5 Properties

Vitamin $B_1$ hydrochloride is soluble in water (1 g in 1 cc) and alcohols (1 g in 100 cc of 95% alcohol or in 315 cc absolute alcohol, or in 18 cc of glycerin), but insoluble in ether, chloroform, benzene and acetone [24] It is optically inactive [30] Vitamin $B_1$ hydrochloride crystallizes from alcoholic aqueous solutions as the hemihydrate (colorless monoclinic needles), melting at 248–250° [42] The bromide hydrobromide hemihydrate occurs as rosettes of needles that melt at 229–231°

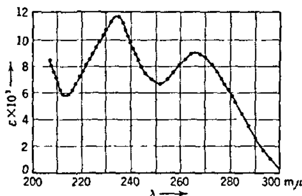



Fig 6 —Absorption spectrum of vitamin $B_1$ (O Wintersteiner R R Williams and A E Ruehle)

The melting points of various other salts are as follows sulfate m p 203° and 276–278° [43] nitrate m p 164–165° [43] picrolonate (dimorphous) m p 165° or 229°, picrate m p 208° gold salt m p 189° rufinate m p 291° [44] All melting points are somewhat uncertain because of attendant decomposition The free vitamin base may be obtained from the chloride in amorphous form by treatment with silver oxide On standing in air of average humidity vitamin $B_1$ crystals absorb humidity in an amount of one mol Vitamin $B_1$ crystals and solutions have a slight yeast like or nutty odor

The ultraviolet absorption spectrum of the vitamin hydrochloride shows two bands at a pH of 7 (or greater) at 235 mμ and 267 mμ, respectively [45]

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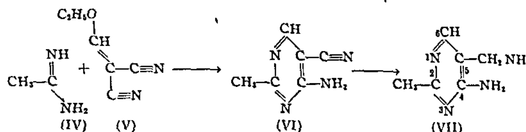
[42] J K Cline R R Williams and J Finkelstein *J Am Chem Soc* 59 1052 (1937) R R Williams and J K Cline *Ibid* 59 216 (1937)

[43] W Kinnersley J R O'Brien and R A Peters *Biochem J* 29 701 (1935)

[44] R R Williams *Ergeb Vitamin Hormonforsch* 1 217 (1938)

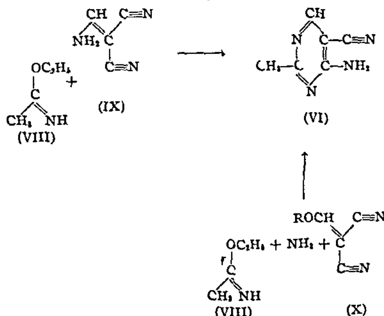
[45] O Wintersteiner R R Williams and A E Ruehle *J Am Chem Soc* 57 517 (1935)

methyl 4 amino 5 amino methyl pyrimidine The synthesis was carried out as follows




Acetamidine (IV) was reacted with ethoxy methylene malonic nitrile (V) yielding 2 methyl-4 amino 5 cyano pyrimidine (VI), which compound was converted into 2 methyl-4 amino 5 amino methyl pyrimidine (VII) by catalytic hydrogenation

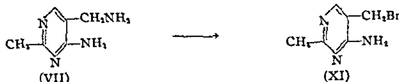
Variations of this synthesis consist in condensing acetimido ethyl ether hydrochloride (VIII) with amino methylene malonic nitrile (IX) to give 2 methyl 4 amino 5 cyano pyrimidine (VI) [64] Or acetimido ethyl ether hydrochloride is condensed with ammonia and ethoxy methylene malonic nitrile (X) [65]



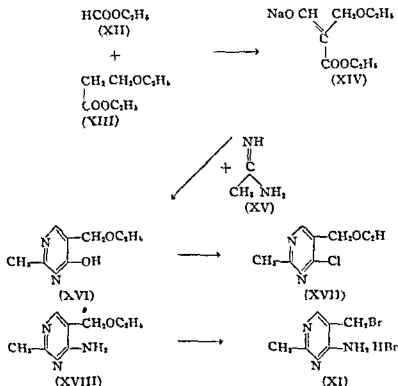

A number of different methods have been worked out for synthesizing 2 methyl-4 amino 5 bromo methyl pyrimidine (XI), which is used in the

[64] O Hromatka G I 667 990
[65] O Hromatka G I 670 635

synthesis of vitamin $B_1$ (see page 116) Grewe converted the 5 amino methyl compound (VII) (see above) into the corresponding 5 bromo methyl compound (XI)[66]




Cline Williams and Finkelstein obtained the compound (XI) in the following way[67] By condensing ethyl formate (XII) and $\beta$ ethoxy ethyl



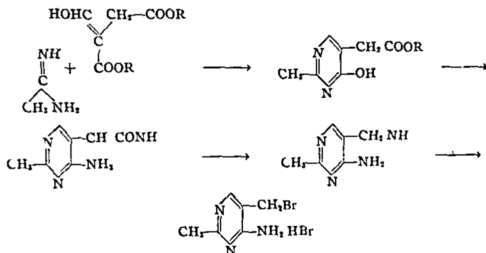

propionate (XIII) with sodium sodium formyl $\beta$ ethoxy ethyl propionate was obtained (XIV) which by condensation with acetamidine hydro chloride (XV) yielded 2 methyl-4 hydroxy 5 ethoxy methyl pyrimidine (XVI) The hydroxyl group in 4 position was converted into the chloride (XVII) by phosphorus-oxychloride and finally into an amino group (XVIII) by ammonia in alcohol By the action of hydrobromic acid the

[66] See also T Imai nd K Makino *Z phy l Ch m* 252 76 (1938)

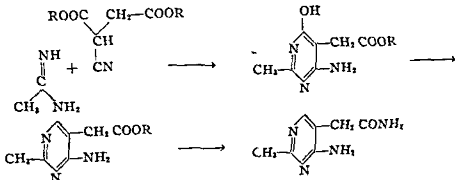
[67] J K Cl ne R R W ll m and J Finkel tein *J Am Chem Soc* 59 10 2 (1937)

ethoxy group in the methyl in 5 position was replaced by bromine, yielding the 2 methyl 4 amino 5 bromo methyl pyrimidine hydrobromide (XI)

Andersag and Westphal have chosen another route [68] By condensation of acetamidine with formyl ethyl succinate, 2 methyl 4 oxypyrimidine 5 ethyl acetate is formed The hydroxyl group is replaced by chlorine and the chloro compound on reaction with liquid ammonia yields 2 methyl-4 amino pyrimidine 5 acetamide By a Hoffmann degradation 2 methyl 4 amino 5 methyl amino pyrimidine is obtained nearly quantitatively By the action of nitrous acid on the diamine, only the aliphatic amino group is converted into the corresponding alcohol Hydrobromic acid treatment yields finally the 2 methyl-4 amino 5 bromo methyl pyrimidine hydro bromide




A modification of this method consists in the condensation[69] of acetamidine with ethyl cyano succinate, yielding 2 methyl-4 amino 6-oxypyrimidine 5 ethyl acetate The hydroxyl group in 6 position is converted into a




[68] H Andersag and K Westphal *Ber* 70 2035 (1937)

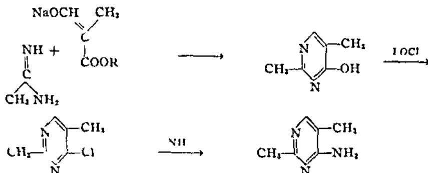
[69] H Andersag and K. Westphal G P 671 787

chlorine group and the chlorine is eliminated by zinc dust yielding the 2 methyl-4 amino pyrimidine 5 ethyl acetate  Ammonia treatment yields then the 5 acetamide compound of the previously mentioned synthesis

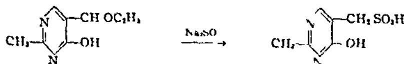
As has been mentioned before the sulfite cleavage product of vitamin $B_1$ that contains the pyrimidine ring has the formula (II)  This acid was synthesized by Grewe[70] from the previously described 2 methyl-4 amino 5 bromo methyl pyrimidine by heating with an acid bisulfite solution




By reduction with sodium in liquid ammonia the amino sulfonic acid (II) gives a base[71] $C_6H_9N_3$ the picrate of which proved to be identical with that of 2,5 dimethyl 4 amino pyrimidine  The latter compound was synthesized[72] by condensing acetamidine and sodium formyl ethyl propionate transforming the obtained 2 5 dimethyl 4 hydroxy pyrimidine into the corresponding 4 chloro compound and exchanging the chlorine with ammonia

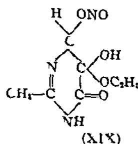



The oxy sulfonic acid, 2 methyl 4 hydroxypyrimidine 5 methyl sulfonic acid, mentioned before as the reaction product of hydrochloric acid with the amino sulfonic acid (II) has been obtained by reacting the synthetically obtained 2 methyl-4 hydroxy 5 ethoxy methyl pyrimidine with sodium sulfite[71]

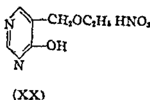



[70] R Grewe Z physiol Chem 242 89 (1936)
[71] J K Cline R R Williams A E Ruehle and R E Waterman J Am Chem Soc 59 530 (1937)
[72] R R Williams A E Ruehle and J Finkelstein Ibid 59 [illegible] (193[illegible])

By mild oxidation of vitamin $B_1$ with nitric acid another pyrimidine derivative is obtained It is an ethyl ester of the formula $C_7H_{11}N_3O_5$ and may have the constitution of (XIX) according to Grewe[73, 74] or of (XX) according to Williams[75]



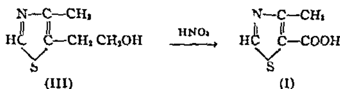
(XIX)

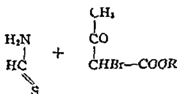


(XX)

### (b) The Thiazole Part

The other reaction product of the sulfite cleavage of vitamin $B_1$ is the base $C_6H_9ONS$ (III) The sulfur in this compound is, in differentiation to the sulfur in the pyrimidine part of the cleavage product, in the same form as in the vitamin By oxidation of the base with nitric acid the side chain is degradated, yielding the acid (I), which is also obtained by direct



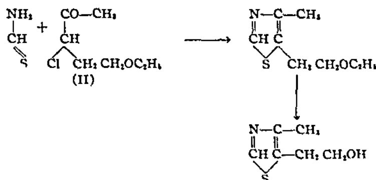

nitric acid oxidation of the vitamin,[76] and is identical with the synthetically[77, 78] prepared 4 methyl thiazole 5 carboxylic acid[79, 80] This syn

[73] A Windaus R Tschesche and R Grewe *Z physiol Chem* 237 98 (1935)
[74] R Grewe *Ibid* 242 89 (1936)
[75] R R Williams *J Am Chem Soc* 58 1063 (1936)
[76] A Windaus R Tschesche and R Grewe *Z physiol Chem* 228, 27 (1928)
[77] M Wohmann *Ann* 259 299 (1890)
[78] M L Tomlinson *J Chem Soc* 1935 1030
[79] H T Clarke and S Gurin *J Am Chem Soc* 57 1876 (1935)
[80] E R Buchman *Ibid* 58 1803 (1936)

thesis can be carried out by condensing thioformamide with α bromo aceto acetic ester

The presence of a thiazole ring in vitamin $B_1$ was indicated by ultra violet absorption studies, by the stability against nitric acid and the loss of sulfur by the action of alkaline plumbite

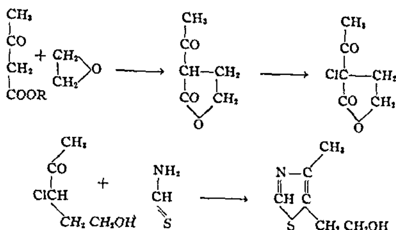
The thiazole part of the sulfite cleavage of vitamin $B_1$ is an alcohol and has one carbon atom more than the acid obtained by its nitric acid oxidation The alcohol is 4 methyl 5 β hydroxy ethyl thiazole (III) The presence of the hydroxyl group was proved[81] by the formation of a chloride and a *p* nitrobenzoate Since this benzoate was found to be basic the presence of a tertiary nitrogen was suspected This was further proved by the formation of a quaternary salt with methyl iodide The primary nature of the alcoholic group was indicated by the result of its nitric acid oxidation by the optical inactivity of the alcohol and the failure of the iodoform test These indications were substantiated by synthesis The alcohol has been synthesized[82] by condensation of thioformamide with the chloro ketone (II) (methyl α chloro γ ethoxy propyl ketone) and hydrolysis to the corresponding alcohol



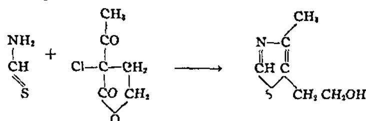

A variety of modifications of this method are also possible Todd and co workers[83 84 85] used instead of the chloro ethyl ether (II) the corresponding chloro ethyl acetate the ester group of which can be saponified with more facility than the ether group Buchman[86] simplified the preparation by condensing acetoacetic acid ester with ethylene oxide to α acetyl butyro lactone chlorinating the lactone in α position with sulfuryl

[81] F R Buchman R R Williams and J C Keresztesy *J Am Chem Soc* 57 1849 (1935)
[82] H T Clarke and S G ... *Ibid* 57 1876 (1935)
[83] G Barger F Bergel and A R Todd *Nature* 136 259 (1935) *Ber* 68 2257 (1935)
[84] A R Todd F Bergel and Karimullah *Ber* 69 217 (1936)
[85] A R Todd F Bergel Karimullah and R Keller *J Chem Soc* 1937 361 A R Todd F Bergel and A Jacob *Ibid* 1936 1555
[86] F R Buchman *J Am Chem Soc* 58 1803 (1936)

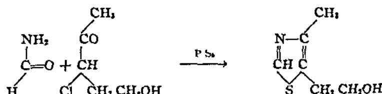
chloride and obtaining by hydrolysis $\gamma$ chloro $\gamma$ acetyl propyl alcohol, which by condensing with thioformamide yielded the 4 methyl 5 $\beta$ hydroxy ethyl thiazole



A further simplification was made by Wenz,[87] who found that the $\alpha$ chloro $\alpha$ acetyl butyro lactone can be condensed with thioformamide directly to the thiazole compound



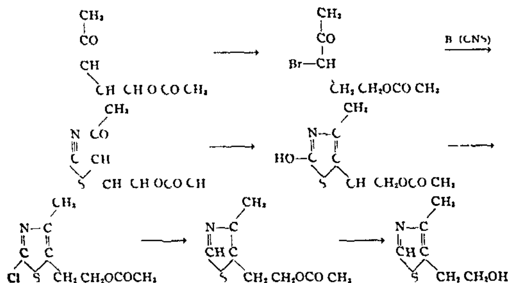
Another modification, by Hromatka,[88] was the condensation of formamide with $\gamma$ chloro $\gamma$ acetyl propyl alcohol in the presence of phosphorus pentasulfide



A different synthesis was announced by Andersag and Westphal[89] $\gamma$ aceto propyl acetate is brominated and the bromo compound condensed with barium thiocyanate, forming $\gamma$ thiocyano $\gamma$ aceto propyl acetate This rearranges in acid solution to the isomeric 2 hydroxy-4 methyl 5

[87] A Wenz G P 664 789
[88] O Hromatka U S P 2 160 867
[89] H Andersag and K. Westphal *Ber* 70 2035 (1937)

acetoxy ethyl thiazole The hydroxyl group in 2 position is converted into chlorine by phosphorus oxychloride and the chloride by means of zinc and acetic acid is transformed into 4 methyl 5 acetoxy ethyl thiazole which by saponification yields 4 methyl 5 $\beta$ hydroxy ethyl thiazole



(c) *The Connection of the Pyrimidine and the Thiazole Part*

The pyrimidine compound and the thiazole compound are connected in the vitamin $B_1$ molecule by a methylene bridge The presence of the methylene group was suspected by the ultraviolet absorption of the vitamin [90] and was proved by synthesis The pyrimidine portion is connected with the carbon atom to which the sulfonic acid group is added by the sulfite cleavage process The thiazole portion is connected with the tertiary nitrogen atom, since in the vitamin itself the nitrogen is quaternary This has been shown by the already mentioned addition of methyl iodide to the thiazole compound and by potentiometric titrations of the quaternary methyl iodide compound and of the vitamin hydrochloride[91-94] (see Fig 7)

Williams states [95] On titrating thiamin hydrochloride with alkali, we find a sharp rise when 1 mol is reached corresponding to the formation of the neutral or mono acid salt but there is no further break until a total of

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[90] K. Makino and T. Imai *Z physiol Chem* 239 1 (1936)
[91] H. T. Clarke and S. Gurin *J Am Chem Soc* 57 1876 (1935)
[92] T. W. Birch and L. J. Harris *Nature* 135 654 (1935)
[93] R. G. C. Moggridge and A. G. Ogston *Biochem J* 29 866 (1935) A. G. Ogston and R. A. Peters *Ibid.* 30 736 (1936)
[94] R. R. Williams and A. E. Ruehle *J Am Chem Soc* 57 1856 (1935)
[95] R. R. Williams and T. D. Spies *Vitamin $B_1$* New York 1938 p 163

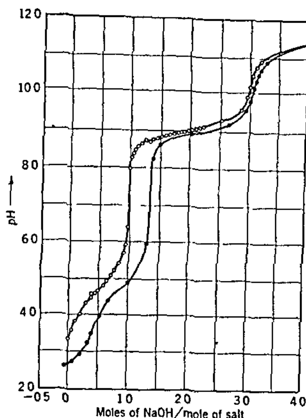
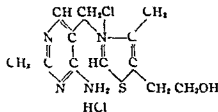


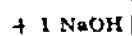

Fig 7 Titration of vitamin $B_1$ chloride with NaOH (o—o) and back titration with HCl (●—●) (R R Williams and A E Ruehle)

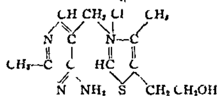
3 mols has been added Apparently the addition of 1 mol of alkali liberates the monochloride (*b*) Further addition of alkali begins to liberate the strong quaternary base (*c*) which goes over rapidly into the neutral pseudo base or carbinol (*d*) This does not occur instantaneously, as one can see during titration by the momentary rise and subsequent slow fall of pH after each addition of alkali Such process of the migration of hydroxyl is well known in cyclic quaternary bases and in effect uses up hydroxyl ions Nor does the pH rise even when 2 mols have been added because quaternary thiazoles such as this undergo ring opening in alkali solution, forming an acidic sulfhydryl group[96] Only after this is neutralized with a third mol of alkali to form (*e*) does the alkalinity rise sharply, indicating free NaOH The reverse arrows indicate reversal of the sequence and the regeneration of the vitamin upon back titration If the solution stands in alkaline condition for some time, there is some permanent destruction of the vitamin

[96] W H Mills I M Clark and J A Aeschlimann *J Am Chem Soc* 123 ?3 3 (1923)

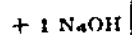


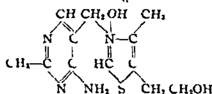
(a)



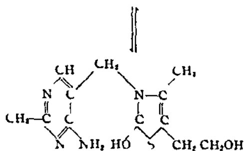



(b)



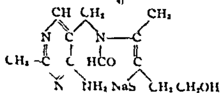



(c)



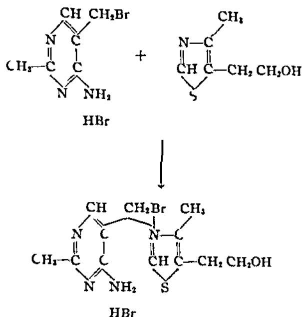
(d)



(e)

The final proof for the connection of both fragments is given by the total synthesis of vitamin $B_1$. 2 Methyl 4 amino 5 bromo methyl pyrimidine hydrobromide upon condensation with 4 methyl 5 $\beta$ oxy ethyl thiazole yields the vitamin $B_1$ bromide hydrobromide [97, 98, 99]




The bromide hydrobromide can be converted into the chloride hydrochloride either by shaking with silver chloride[98] or by preparing the sparingly soluble picrate followed by digestion with 10% hydrochloric acid [99]

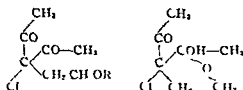
An improvement[100] in this method consists of condensing the hydrochloride of the 5 hydroxymethyl pyrimidine compound with the hydrochloride of the thiazole compound, giving the vitamin chloride hydrochloride directly

An entirely different method of synthesizing vitamin $B_1$ has been effected[101, 102] by condensing 2 methyl 4 amino 5 thioform amido methyl pyrimidine, which can be obtained from the 5 amino methyl compound by condensation with potassium dithio formate or with ethyl formate in the presence of phosphorus pentasulfide with $\gamma$ chloro(or bromo) $\gamma$ aceto propyl alcohol (or acetate or benzoate)

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[97] R R Williams and J K Cline *J Am Chem Soc* 58 1504 (1936)
[98] J K Cline R R Williams and J Finkelstein *Ibid* 59 1052 (1937)
[99] H Andersag and K Westphal *Ber* 70 2035 (1937)
[100] O Zima G P 669 187
[101] A R Todd and F Bergel *J Chem Soc* 1937 364
[102] H Andersag and K Westphal *Ber* 70 2035 (1937)

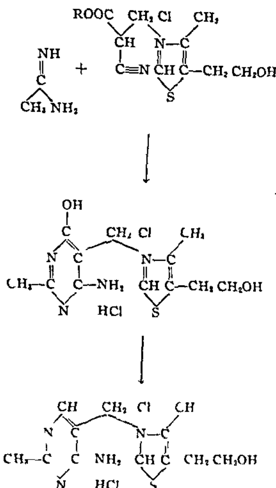
Instead of the γ halogen γ aceto propyl alcohol also γ γ diaceto γ halogeno (or mercapto) propyl alcohol or its inner hemiacetale might be used [103]




A modification of this method which is said to give much better yields[104] consists in the condensation of 2 methyl-4 amino 5 thioform amido methyl pyrimidine with 2 methyl 2 alkoxy 3 chloro tetrahydrofurane which can be obtained from the previously described α chloro α aceto butyro-lactone and a primary alcohol in the presence of sulfuric acid

[103] T M t kawa nd M Oht U S 2 184 7 0 Ind an 75 809
[104] M Kl ngenfuss U S P 2 127 446 G P 676 950 F P 831 110

A different method of synthesizing vitamin $B_1$ should finally be mentioned[105] It consists in building the pyrimidine nucleus on the thiazole nucleus and is best described by the following series of formulas




## 7 Industrial Methods of Preparation

Vitamin $B_1$ is manufactured today by total synthesis according to the methods described before The isolation from natural sources which is more expensive is however, still carried out technically The latter method has been worked out systematically[106] The vitamin is adsorbed on fuller s earth or synthetic zeolites[107, 108, 109] extracted with acid salts

[105] H Andersag and K Westphal F P 816 432
[106] R D Greene and A Black *J Am Chem Soc* 59 1395 (193 )
[107] L R Cerecedo and D J Hennessy *Ibid* 59 1617 (1937)
[108] L R Cerecedo and F J Kaszuba *Ibid* 59 1619 (1937)
[109] L R Cerecedo and J J Thornton *Ibid* 59 1621 (1937)

of organic nitrogen containing bases such as pyridine or quinine the adventitious matter removed by benzoylation in soda alkaline solution and chloroform extraction, followed by precipitation of the vitamin with silver nitrate barium hydroxide and phosphotungstic acid Vitamin $B_1$ is finally recrystallized from acidified organic solvents, such as combinations of phenol and butyl alcohol or HCl alcohol From one ton of rice polishings 5–10 g of vitamin $B_1$[110] can be obtained by this method

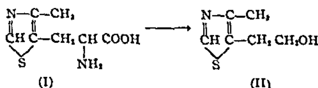
Commercial vitamin $B_1$ contains about 4% water which can be removed by drying at 100° C or by standing in a vacuum over sulfuric acid Elixirs of thiamin chloride are marketed which contain a wine base Since tannic acid precipitates vitamin $B_1$ the wine is completely freed from tannic acid by the use of freshly prepared casein or completely defatted and dealbuminized milk [111]

The preparation of the vitamin $B_1$ pyrophosphate, which will be described later is also commercially important

## 8 Biogenesis

The natural precursors of vitamin $B_1$ and the reactions leading to the synthesis of vitamin $B_1$ in the plant organism are unknown The hypothesis has been advanced[112, 113] that the particular thiazole part of the vitamin $B_1$ molecule might arise during yeast fermentation from $\alpha$ amino $\beta$ (4 methyl thiazole 5) propionic acid (I), which could have been built up from methionine acetaldehyde and ammonia, by a reaction analogous to the formation of fusel oil in alcoholic fermentation Yeast has been found to be able to convert this amino propionic acid derivative of thiazole (I) into the 4 methyl 5 ($\beta$ hydroxy ethyl) thiazole (II) of vitamin $B_1$

(For the influence of light and chlorophyll upon the vitamin $B_1$ synthesis and for the site of the synthesis in higher plants see page 133)




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[110] R R Williams R E Waterman and J C Keresztesy *J Am Chem Soc* 56 1187 (1934)

[111] I Greenegard *J Am Pharm Assoc* 1 230 (1940)

[112] C R Harington and R C G Moggridge *Biochem J* 34 685 (1940) *J Chem Soc* 1939 443

[113] J Bonner and F R Buchman *Proc Natl Acad Sci U S* 24 431 (1939)

## 9 Thiochrome

By oxidation, vitamin $B_1$ is converted into thiochrome a yellow substance of intense blue fluorescence [114] This conversion occurs also when alcoholic solutions of the vitamin stand at room temperature for several months [115] Very little thiochrome is formed at pH 2, but it is produced more rapidly as the pH approaches 7 Thiochrome can be obtained from vitamin $B_1$ by oxidation with permanganate or manganese oxides at pH 7,[116] by oxidation of alkaline solutions of vitamin $B_1$ with potassium ferricyanide,[117] by hydrogen peroxide, selenium dioxide, etc [118] The same substance has been isolated from yeast by Kuhn and co workers[119] who proposed the name thiochrome The compound melts at 227–228° and exhibits absorption maxima at 358 and 375 mμ (see Fig 8) The isolation of thio

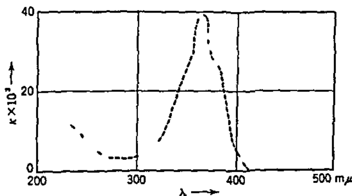
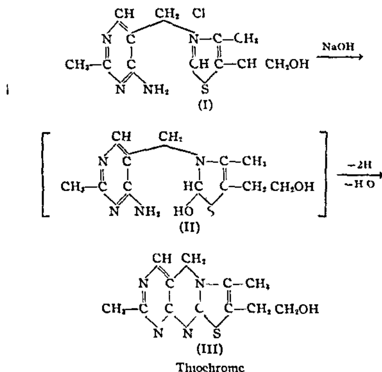



Fig 8 —Absorption spectrum of thiochrome (R Kuhn and H Vetter)

chrome has been carried out by adsorption on acid silicates, followed by elution and precipitation with silver nitrate From 8100 lbs yeast, about 250 mg thiochrome are obtained Thiochrome probably does not occur as such in yeast, but is formed when the yeast is worked up Sodium hydrosulfite reduces thiochrome in neutral or alkaline solutions to a leuco compound which does not fluoresce By air oxidation thiochrome is regenerated In alkaline solutions thiochrome is sensitive to light and the

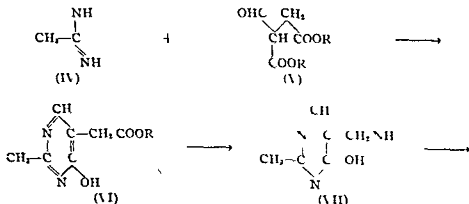
[114] R A Peters *Nature* 135 107 (1935)
[115] H W Kinnersley J R O Brien and R A Peter *Biochem J* 29 701 (1935)
[116] H W Kinnersley J R O Brien and R A Peters *Ibid* 29 2369 (1935)
[117] G Barger F Bergel and A R Todd *Nature* 136 259 (1935) *Ber* 68 2257 (1935)
[118] R Kuhn and H Vetter *Ber* 68 2375 (1935)
[119] R Kuhn T Wagner Jauregg F W von Klaveren and H Vetter *Z physiol Chem* 234 196 (1935)

fluorescence disappears irreversibly The mechanism of the dehydrogenation of vitamin $B_1$ to thiochrome is not known with certainty It is however, very probable that at first an intermediate is formed of the following formula (II)

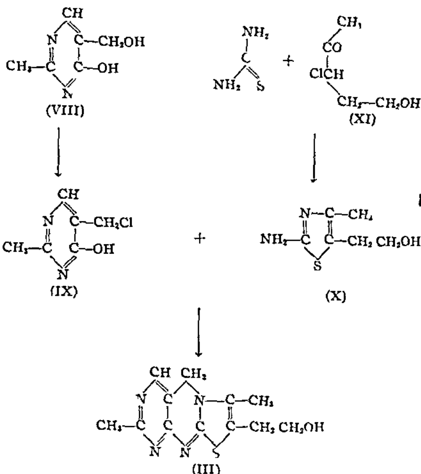


Thiochrome

Thiochrome has been prepared synthetically in the following manner [120]




[120] A R Todd F Bergel H L Fraenkel Conrat and A Jacob J Chem Soc 1936 1601




Condensation of acetamidine (IV) with formyl succinate (V) yielded 4 hydroxy 2 methyl pyrimidine 5 ethyl acetate (VI) from which by Curtius degradation 4 hydroxy 5 amino methyl 2 methyl pyrimidine (VII) was obtained Replacement of the amino group by hydroxyl was effected by means of nitrous acid and the resulting 4 hydroxy 5 hydroxy methyl 2 methyl pyrimidine (VIII) on boiling with phosphorus chloride yielded the chloro compound (IX) 2 Amino 4 methyl 5 $\beta$ hydroxy ethyl thiazole (X) was obtained by condensing methyl $\alpha$ chloro $\gamma$ hydroxy propyl ketone (XI) with thiourea The pyrimidine and the thiazole compound form thio chrome upon condensation

Compounds with the same ring skeleton as thiochrome are called quino chromes

## 10 Vitamin $B_1$-Pyrophosphate

The biological action of vitamin $B_1$ is partly or mainly due to the action of its pyrophosphoric acid ester Vitamin $B_1$ apparently acts as an ac

By enzymatic dephosphorylation with prostata phosphatase or with a phosphatase which is liberated from yeast cells during drying,[128] free vitamin $B_1$ is obtained. Only one molecule of phosphoric acid is hydrolyzed by the action of alkaline kidney phosphatase[129, 130] or by acid hydrolysis. The second mol of phosphoric acid is much more difficult to hydrolyze. Neither the monophosphoric acid obtained nor vitamin $B_1$ shows cocarboxylase action.

The chemical reactions of cocarboxylase resemble closely those of the free vitamin $B_1$. By slight oxidation, a blue fluorescent compound of the thiochrome type is obtained. By the action of sulfite the molecule is split as described for the vitamin itself, into the pyrimidine part $C_6H_9O_3N_3S$ and the diphosphorylated thiazole compound.

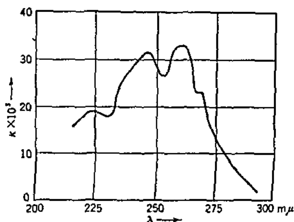



Fig. 9.—Absorption spectrum of vitamin $B_1$ pyrophosphate (cocarboxylase). (H. Rudy in W. Stepp, *Ernahrungslehre*.)

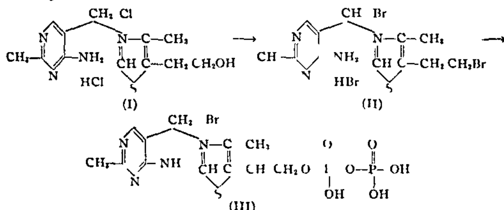
Vitamin $B_1$ pyrophosphate in aqueous solution forms an inner salt connecting the amino group of the pyrimidine part with one of the hydroxyl groups of the phosphoric acid part. The absorption spectrum of vitamin $B_1$ pyrophosphate (see Fig. 9) resembles closely that of the free vitamin.

The synthesis of cocarboxylase has been achieved by enzymatic and by chemical methods. Phosphatase[131] of the duodenal mucosa of the pig, dried brewer's yeast and living yeast[132] were successfully employed as enzymatic agents for the phosphorylation of vitamin $B_1$ in the presence of

[128] D. Melnick and H. Field, *J. Biol. Chem.* 127, 531 (1939).
[129] K. Lohmann and P. Schuster, *Biochem. Z.* 294, 188 (1937).
[130] H. Tauber, *J. Biol. Chem.* 123, 499 (1938).
[131] H. Tauber, *Science* 86, 180 (1937); *Enzymologia* 2, 171 (1937); *J. Biol. Chem.* 123, 499 (1938).
[132] H. W. Kinnersley and R. A. Peters, *J. Soc. Chem. Ind.* 56, 447 (1937); *Biochem. J.* 32, 697 (1938).
M. A. Lipschitz, Van R. Potter and C. A. Elvehjem, *Biochem. J.* 32, 474 (1938).

hexose diphosphate and adenine triphosphate [133] Phosphoglyceric acid and phosphopyruvic[134] acid can also act as phosphate donator in the presence of catlaytic amounts of adenylic acid or adenyl pyrophosphate [135] Cocarboxylase is also enzymatically prepared from vitamin $B_1$ monophosphate which apparently is first hydrolyzed to the non phosphorylated vitamin [135] All these enzymatic processes occur only in the presence of the apocarboxylase the specific protein of the carboxylase and are stopped when the protein is partly or fully saturated [134, 135] Therefore no preparative use can be made of the enzymatic synthesis of cocarboxylase Cocarboxylase has also been obtained from vitamin $B_1$ by bacterial synthesis using *Propionibacterium pentosaceum* [136]

Chemically, cocarboxylase was synthesized from vitamin $B_1$ in low yields by the action of phosphorus oxychloride [137] with better yields by condensation with sodium pyrophosphate in the presence of phosphoric acid[138] or by the use of pyrophosphoryl chloride [139] Another method consists in the conversion of vitamin $B_1$ (I) into the bromo vitamin (II) by the action of hydrobromic acid The bromo compound in turn is reacted with a solution of silver pyrophosphate in pyrophosphoric acid to yield the cocarboxylase (III) [140] For the purification of the synthesized cocarboxylase chloride a precipitation with phosphotungstic acid has been recommended and final crystallization is achieved from alcoholic HCl [141]




[133] H v Euler and R Vestin *Naturwissenschaften* 25 416 (1937)

[134] M A Lipton and C. A Elvehjem *Cold Spring Harbor Symposia on Quant Biol* 7 (1939) *Nature* 145 226 (1940)

[135] H Weil Malherbe *J Soc Chem Ind* 58 1071 (1939)

[136] M Silverman and C H Werkman *Proc Soc Exptl Biol Med* 40 369 (1939)

[137] K. G Stern and J W Hofer *Enzymologia* 3 82 (1937)

[138] J Weijlard and H Tauber *J Am Chem Soc* 60 730 2263 (1938)

[139] K Lohmann quoted in C Oppenheimer and K G Stern *Biological Oxidation* New York 1939 p 207

[140] H Weil Malherbe *J Soc Chem Ind* 58 1071 (1939) *Biochem J* 34 980 (1940)

[141] J Weijlard *J Am Chem Soc* 63 1160 (1941)

Cocarboxylase as well as the vitamin monophosphate have the biological activity of the vitamin $B_1$ [142]

The chemistry of the apocarboxylase, the protein part of the carboxylase is not known The molecular weight is about 150,000 [143]

## 11 Specificity of the Vitamin $B_1$ Action

The vitamin action of vitamin $B_1$ seems to be connected with the specific structure of the molecule The different vitamin salts, as the hydrochloride, hydrobromide,[144] sulfate etc , and the pyrophosphoric acid ester, cocarboxylase have a corresponding activity The vitamin $B_1$ *disulfide (I)* is also as active as the vitamin

(I)

Structural alterations, however, cause disappearance of the vitamin action Inactive are thiochrome [145, 146] the *oxychlorothiamin*[147] (II) and the products obtained from the sulfite cleavage of the vitamin [148, 149] Dihydro

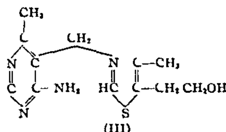
(II)

vitamin $B_1$ is also inactive, but the *dihydro* cocarboxylase was found to be active [150] Polyneuritis in pigeons may however, be cured[151] by 4 amino

[142] K Lohmann and P Schuster *Biochem Z* 294 188 (1937)
[143] C Oppenheimer and K G Stern *Biological Oxidation* New York 1939 p 208
[144] R R Williams and J K Cline *J Am Chem Soc* 58 1504 (1936)
[145] G Barger F Bergel and A R Todd *Nature* 136 59 (1935) *Ber* 68 2257 (1935)
[146] R Kuhn and U Vetter *Ber* 68 2375 (1935)
[147] E R Buchman and R R Williams *J Am Chem Soc* 57 1751 (1935)
[148] R R Williams *Ibid* 57 229 (1935)
[149] R R Williams R E Waterman J C Keresztesy and E R Buchman *Ibid* 57 536 (1935)
[150] C Oppenheimer and K G Stern *Biological Oxidation* New York 1939 p 204
[151] W J Robbins M A Bartley A G Hogan and E R Richardson *Proc Natl Acad Sci U S* 23 388 (1937)

2 methyl 5 bromo methyl pyrimidine and 4 methyl 5 β hydroxy ethyl thiazole if given simultaneously (A synthesis of vitamin $B_1$ probably occurs from the intermediates)

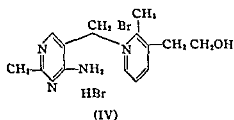
A great number of compounds have been prepared synthetically[15] with slight differences in the vitamin structure Only one (III) has been found active and that one only in much larger doses [153]



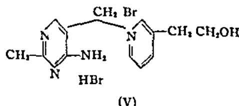
(III)

The pyrimidine ring the thiazole ring and the methylene bridge between them, an unsubstituted amino group in 4 position of the pyrimidine ring,[154] the 5 hydroxy alkyl group[155] and a free 2 position in the thiazole nucleus are all necessary for the vitamin action It is also probable that the nature of the substituents in position 6 influences the vitamin activity [15]

So far as is known only one substance with vitamin $B_1$ properties occurs naturally Growth action of *Phycomyces* is however also obtained by the 2 ethyl instead of the 2 methyl derivative [156] Hetero vitamin $B_1$ (IV) containing instead of the thiazole ring a pyridine ring has an activity of about $^1/_{20}$ of that of vitamin $B_1$ [157]



(IV)



(V)

The next lower homolog (V) devoid of the methyl group in the pyridine ring is only $^1/_{10}$ as active as the methylated product [158]

[153] F Bergel and A R Todd *J Chem Soc* 1937 1504
[154] H Andersag and K Westphal *Ber* 70 2035 (1937)
[155] F Bergel and A R Todd *J Chem Soc* 140 1504 (193 )
[156] D Price and F D Pickel *J Am Chem Soc* 63 1067 (1941)
[157] W J Robbins and F Kavanagh *Proc Natl Acad Sci U S* 24 229 (1938)
[15] F C Schmelkes *Science* 90 113 (1939) F C Schmelkes and R R Joiner *J Am Chem Soc* 61 2562 (1939) P Baumgarten and A Dornow *Ber* 73 44 (1940)
[158] A. Dornow *Ber* 73 156 (1940)

It may be noted that substances of the cyclo pentano perhydro phenanthrene type (for example male and female sex hormones vitamin D) have been reported[159] as delaying the vitamin $B_1$ avitaminosis (polyneuritic symptoms in pigeons) These results however need confirmation

## 12 Determination

### (a) Chemical Methods

A number of color reactions have been proposed for the determination of vitamin $B_1$ Reliable quantitative results can be obtained with the first three procedures when carefully controlled conditions are observed

1 **Formaldehyde-azo-test of Kinnersley and Peters**[160] Diazo benzene sulfuric acid in carbonate containing sodium hydroxide solution gives with vitamin $B_1$ and formaldehyde a red color Since this reaction attacks the 4 amino group of the pyrimidine nucleus, it is obvious that a deaminated vitamin or thiochrome does not give this reaction Therefore this reaction is quite valuable for the determination of the natural vitamin Synthetic compounds of similar structure give the same reaction Substituents in the thiazolium nucleus influence the reaction[161] This method of vitamin $B_1$ determination is applicable to rather highly purified solutions only The presence of reducing substances interferes with the color development The use of acetone in place of vitamin $B_1$ also gives the same color with the reagents of this test method[162]

2 **Thiochrome Test by Jansen** Vitamin $B_1$ in aqueous solution is oxidized by means of potassium ferricyanate to thiochrome, the fluorescence of which is determined photoelectrically after extraction of the thiochrome with isobutanol[163, 164, 165, 166, 167] The oxidation is carried out at pH 10 The intensity of the fluorescence is a function of the alkalinity of the solution and of the amount of thiochrome present Excess potassium ferricyanide destroys the formed thiochrome rapidly *Irradiation also* destroys the thiochrome

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[159] J Sanchez Rodriguez and J M S rda *Z Vitamınforsch* 6 193 (1937)
[160] H W Kinnersley and R A Peters *Biochem J* 28 667 (1934) *Ibid* 32 1516 (1938)
[161] F Bergel and A R Todd *J Chem Soc* 1937 1504
[162] C R Addinall *The Story of Vitamin $B_1$* Merck & Co 1937 p 18
[163] G Barger F Bergel and A R Todd *Nature* 136 259 (1935) *Ber* 68 2 57 (1935)
[164] B C P Jansen *Rec trav chim* 55 1046 (1936) H G K Westenbrink and J Goudsmit *Ibid* 56 803 (1937) P Karrer and U Kubli *Helv Chim Acta* 20 369 (1937) I Widenbauer O Huhn and C Becker *Z ges exptl Med* 101 178 (1937) J Goudsmit and H G K Westenbrink *Nature* 139 1108 (1937) K Ritsert *Deut med Wochschr* 64 481 (1938) H Otto and F Rühmekorb *Klin Wochschr* 17, 1246 (1938)
[165] H G K Westenbrink and J Goudsmit *Nature* 142 150 (1938)
[166] C M Hills *Biochem J* 33 1966 (1939)
[167] R G Booth *J Soc Chem Ind* 59 181 (1940)

The results obtained with this method are in fair agreement with those obtained by the biological bradycardia method It must however be noted that only the free vitamin $B_1$ is determined by this method, since only the free thiochrome can be extracted with organic solvents This difficulty can be overcome by digesting the sample with pepsin followed by digestion with taka diastase [168] Ambiguity arises sometimes through colored fluorescence of other compounds which obscures the effect of thiochrome [169]

3 Colorimetric Method by Prebluda and McCollum [170] Diazotized $p$ amino acetanilide $p$ amino acetophenone or methyl $p$ amino acetophenone gives with vitamin $B_1$ red dyes, specific for this vitamin, which can be extracted with organic solvents such as xylene acetone, isobutanol etc The sensitivity of this reaction is increased by the presence of phenol or ethyl alcohol or preferably, both [171] Willstaedt[172] suggests the use of 2,4 dichloro benzene diazonium chloride extraction of the formed yellow red dye with ether and separation from by products by adsorption on calcium hydroxide This method has been modified by running the analysis with diazotized sulfanilic acid with and without the presence of potassium ferricyanide Since vitamin $B_1$ does not give a color under these conditions but other amino compounds which may accompany the vitamin do give colors, the difference between the color values gives a measure of the amount of vitamin $B_1$ present [173]

The results of these methods compare favorably with the bioassay values Vitamin C interferes with the development of the color [174] unless it is first oxidized, for example, by titrating with iodine or by the addition of calcium ions [175] The phosphorylated forms of vitamin $B_1$ give the color but the dye formed cannot be extracted by organic solvents such as xylene [176]

4 Gravimetric Method by Naiman [177] This method is based on the production of an orange red precipitate of vitamin $B_1$ with bismuth potassium iodide

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[168] M Pyke *J Soc Chem Ind* 58, Trans 338 (1939)

[169] M Pyke *J Soc Chem Ind* 58 1021 (1939)

[170] H J Prebluda and E V McCollum *Science* 84 488 (1936) *J Biol Chem* 127 495 (1939) D Melnick and H Field *J Biol Chem* 127 505 515 (1939)

[171] D Melnick and H Field *J Biol Chem* 127 515 (1939)

[172] H Willstaedt *Naturwissenschaften* 25 68? (1937)

[173] P Meunier and C P Blancpain *Compt rend* 208 768 (1939)

[174] A D Emmett G Peacock and R A Brown *J Biol Chem* 135 131 (1940)

[175] M E Auerbach *J Am Pharm Assoc* 29 313 (1940)

[176] D Melnick and H Field *J Biol Chem* 127 531 (1939)

[177] B Naiman *Science* 85 290 (193 )

5 **Method of Spruyt**[178] Vitamin $B_1$ is separated as the phospho tungstate and reduced with nascent hydrogen The resulting brown color is believed to be proportional to the amount of vitamin $B_1$ present

6 **Raybin Reaction**[179] Vitamin $B_1$ in a borax solution of pH 9 6 forms with 2,6 dibromo quinone chloroimide an *orange color* which gradually decreases in intensity The color formed can be extracted with chloroform and measured in a photometer

7 **Tauber Reaction**[180] Vitamin $B_1$ and *p* dimethyl amino benzaldehyde in the presence of acetic acid produce upon evaporation of the acid and addition of fresh acid an intense brick red color This reaction should not be carried out in the presence of proteins or amino acids which interfere

*Vitamin $B_1$ in the presence of vitamin $B_1$ pyrophosphate* can be determined by either the thiochrome method (thiochrome pyrophosphate cannot be extracted from an alkaline solution with isobutanol)[181] or by condensation with diazo compounds according to Prebluda and McCollum Vitamin $B_1$ pyrophosphate gives the same color reactions as does vitamin $B_1$ The color developed cannot however, be extracted by organic solvents For the determination of the *total vitamin $B_1$* present, the material might be hydrolyzed enzymatically, followed by the determination of the then free vitamin $B_1$ [181, 18]

## (b) *Biological Methods*

More accurate than any of the described chemical methods for the determination of vitamin $B_1$ are the biological methods Either birds, especially the pigeon, or rats are used

The curative pigeon test by Kinnersley, Peters and Reader uses the disappearance of tonic spasms and convulsions[183] Also a prophylactic pigeon test has been worked out Rats are recommended by the U S Pharmacopoeia as test animals in the curative method[184, 185] Vitamin $B_1$ deficiency in rats causes convulsions and paralysis of the lower extremities

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[178] J P Spruyt *Chem Weekblad* 27 298 (1930) H W Acton S Ghosh and A Dutt, *Ind J Med Research* 1933 103

[179] H W Raybin *Science* 88 35 (1938)

[180] H Tauber *Ibid* 86 594 (1937)

[181] H G K. Westenbrink and B C P Jansen *Acta Brevia Neerland Physiol Pharmacol Microbiol* 8 119 (1938) H W Kinnersley and R A Peters *Biochem J* 28 667 (1934) 29 2369 (1935) 32 1516 (1938)

[182] J Houston and S K Kon *Nature* 143 558 (1939)

[183] H W Kinnersley R A Peters and V Reader *Biochem J* 19 820 (1928)

[184] F Hofmeister *Biochem Z* 129 477 (1922) M I Smith *U S Pub Health Service Pub Health Repts* 24 116 (1930) E F Cook *J Am Pharm Assoc* 28 267 (1939)

[185] O L Kline C D Tolle and E M Nelson *J Assoc Official Agr Chem* 21 305 (1938)

which are cured by the administration of the vitamin. The rat growth test involves the determination of the minimum amount of the vitamin required for growth and maintenance.[186] The electrocardiographic bradycardia test according to Birch and Harris is based on the decline of the heart rate of rats during avitaminosis[187] and its cure by the administration of the vitamin. Vitamin $B_1$ may also be determined with chicks by a method[188] in which the degree of postponement of fatal polyneuritic symptoms afforded by the test material is compared with that afforded by various levels of synthetic thiamin chloride hydrochloride. Another method using chicks as assay animals determines the rate of growth produced by vitamin $B_1$.[189]

### (c) *Biochemical Methods*

1. The "Catatorulin"[189a] test[190] which is often used measures *in vitro* the uptake of oxygen by brain tissue from avitaminotic pigeons. Addition of vitamin $B_1$ increases proportionately the amount of oxygen consumed.

2. **Yeast Fermentation Test.** The stimulating effect of vitamin $B_1$ on alcoholic fermentation has been developed into a quantitative test.[191, 192] As little as 1 $\gamma$ may be detected ($^1/_3$ International Unit).

3. **Mold Growth Method.** Within certain limits the growth of the mold *Phycomyces blakesleanus* is proportional to the concentration of vitamin $B_1$ present in a synthetic nutritive medium and has been used for the determination of vitamin $B_1$ in blood; 0.01 $\gamma$ can be detected according to this procedure.[193] A modification of this method[194] measures the increase of carbon dioxide formed by the addition of vitamin $B_1$ to growing *Phycomyces* (sensitivity 0.1 $\gamma$).

4. **Cocarboxylase** can be determined quantitatively by the amount of $CO_2$ produced by decarboxylation of pyruvic acid by yeast which has

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[186] H. C. Sherman and A. Spohn, *J. Am. Chem. Soc.* 45, 9719 (19 3). I. F. Chase, *Dissertation*, Columbia, 19 8. H. C. Sherman and S. L. Smith, *The Vitamins*, Chemical Catalog Co., 1931.

[187] T. W. Birch and L. J. Harris, *Biochem. J.* 28, 602 (1934).

[188] T. H. Jukes and H. Heitman, *J. Nutrition* 19, 21 (1940).

[189] A. Arnold and C. A. Elvehjem, *Ibid.* 15, 403 (1938).

[189a] Torulin = vitamin B.

[190] R. Passmore, R. A. Peters and H. M. Sinclair, *Biochem. J.* 27, 842 (1933). H. W. Kinnersley, J. R. O'Brien and R. A. Peters, *Ibid.* 29, 701 (1935). R. A. Peters, *Ibid.* 32, 9031 (1938).

[191] A. S. Schultz, L. Atkin and C. N. Frey, *J. Am. Chem. Soc.* 59, 948, 2457 (1937); 60, 1514 (1938).

[192] A. S. Schultz, L. Atkin and C. N. Frey, *Ibid.* 60, 490 (1938).

[193] W. H. Schopfer, *Z. Vitaminforsch.* 4, 67, 187 (1935). W. H. Schopfer and A. Jung, *Compt. rend. soc. biol.* 122, 749 (1936). A. P. Meiklejohn, *Biochem. J.* 31, 1441 (1937). H. M. Sinclair, *Ibid.* 32, 2185 (1938).

[194] E. Heyn, *Z. physiol. Chem.* 258, 219 (1939).

been freed from cocarboxylase in the presence of maximum amounts of vitamin $B_1$[195]

## 13 Standards

One International Unit vitamin $B_1$ = 3 γ of vitamin $B_1$ hydrochloride, colorless monoclinic plates m p 246–247° (decomp ) = 1 U S Pharmacopoeia Unit

This unit replaces the earlier one of 1934 according to which 10 mg of a special adsorbate of vitamin $B_1$ on fuller s earth represented 1 International Unit The new Unit is the biological equivalent of the old Unit The adsorption product which represented the old standard was prepared as follows 100 kg rice polishings are extracted with water sufficient sulfuric acid being added to obtain a pH of 4 5 Salicylic acid (0 2%) and toluene are added to prevent bactericidal decomposition After two days extraction the solution is filtered and shaken for 24 hours with especially adsorptive fuller s earth After filtration the fuller s earth is dried

In order to standardize vitamin $B_1$ preparations of unknown activity, the U S Pharmacopoeia recommends the rat curative method (see above)[196]

1 I U vitamin $B_1$ corresponds about to[197] 0 5 Smith Curative Unit[198] 2 0 Chase Sherman Units[199] 1 0 Roscoe Unit[200] and 20 0 Milligram Equivalents (Cowgill)[201] Units other than I U should no longer be used Actually it is impossible to translate one unit system into another by factors or by mathematical computation The above figures are given only as a guide to indicate the approximate order of magnitude for the purpose of facilitating the reading of some of the original literature

## 14 Physiology of Plants and Microorganisms[202]

Thiamin is a vitamin with respect to animal nutrition Its occurrence in plants and microorganisms has raised the question as to the physiological significance of this compound in plants and microorganisms This question can partly be answered Vitamin $B_1$ is a true growth promoting substance for such organisms and is needed in small amounts for normal development

Plants and microorganisms can be classified into two groups namely those which need an external supply of the growth promoting substance

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[196] S Ochoa and R A Peters *Biochem J* 32 1501 (1938)
[196] F F Cook *J Am Pharm Assoc* 28 267 (1939)
[197] C R Addinall *The Story of Vitamin B1* Merck Co 1937
[198] M I Smith *U S Pub Health Service Pub Health Repts* 24 11t (1930)
[199] H C Sherman and A Spohn *J Am Chem Soc* 45 2719 (1923) F F Chase *Dissertation* Columbia 1928 H C Sherman and S I Smith *The Vitamins* Chemical Catalog Co 1931
[200] H Chick and M H Roscoe *Biochem J* 23 498 (1929)
[201] G R Cowgill *The Vitamin B Requirement of Man* Yale University Press 1934
[202] W J Robbins *Science* 89 303 (1939)

and those which are able to synthesize it Among the lower plants and microorganisms which have the power of synthesizing vitamin $B_1$ are yeasts bacteria fungi etc Special examples are *Aspergillus niger Agaricus campestris, Absidia glauca, Bacterium coli Bacillus pyocyaneus*, *Chlamydomonas*, *Chlorogonium* and *Polytoma obtusum* (see also page 101) Since many of these organisms contain no chlorophyll, it must be concluded that chlorophyll is not an essential factor for the synthesis of the vitamin It seems significant, however that plants (peas) raised in the dark contain very little thiamin whereas the content of this compound in plants increases rapidly in light [203] All higher plants are able to synthesize thiamin It has been demonstrated for example, for tomatoes that the vitamin is synthesized in the shoots In leaves the concentration of vitamin $B_1$ amounts to a constant value of about 25 International Units per 100 g regardless of the botanical family [204] Roots do not have the power of synthesizing vitamin $B_1$ however they need thiamin for continued cell division in the embryonic region Vitamin $B_1$ is essential for the growth of all species of roots investigated [205] Thus thiamin appears to be a true plant hormone On the other hand tomato roots are able to synthesize the pyrimidine portion of thiamin, but require an external supply of the thiazole part (Experiments done with excised tomato roots ) Pea roots require an external supply of both parts [206] Vitamin $B_1$ is stored in seeds and more specifically in the outer integuments

Some plant species (for example, cosmos camellia etc ) are said to grow more luxuriantly when the roots obtain besides the self synthesized vitamin $B_1$ some of this vitamin from an outside source Under very carefully conducted experiments it has not however, been possible to detect any beneficial effects from added vitamin $B_1$ when plants were raised from their seeds [207] It has been shown that in cuttings and after transplanting vitamin $B_1$ promotes root growth [208]

Higher plants not only synthesize vitamin $B_1$ but excrete it through the roots Therefore the soil in the immediate vicinity of plant roots supports a much higher microbial population than that existing outside the plant s

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[203] J Bonner and J Greene *Botan Gaz* 100 6 (1938)

[204] M Pyke *Biochem J* 34 330 (1940)

[205] J Bonner *Am Chem Soc Div Agr Food Chem Meeting* Sept 1939 Abst 13 F W Went J Bonner and G C Warner *Science* 87 170 (1938)

[206] F Kögl and A J Haagen Smit *Z physiol Chem* 243 209 (1936) J Bonner *Science* 85 183 (1937) W J Robbins and M A Bartley *Ibid* 85 246 (1937) W J Robbins M A Bartley A G Hogan and L R Richardson *Proc Natl Acad Sci U S* 23 385 (193 )

[207] D I Arnon *Science* 92 264 (1940)

[208] J Bonner *Am Chem Soc Div Agr Food Chem Meeting* Sept. 1939 Abst 13 F W Went J Bonner and G C Warner *Science* 87 170 (1938)

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[195] S Ochoa and R A Peters *Biochem J* 32 1501 (1938)
[196] F F Cook *J Am Pharm Assoc* 28 267 (1939)
[197] C R Addinall *The Story of Vitamin $B_1$* Merck Co 1937
[198] M I Smith *U S Pub Health Service Pub Health Repts* 24 116 (1930)
[199] H C Sherman and A Spohn *J Am Chem Soc* 45 2719 (1923) F F Chase *Dissertation* Columbia 1928 H C Sherman and S I Smith *The Vitamins* Chemical Catalog Co 1931
[200] H Chick and M H Roscoe *Biochem J* 23 498 (1929)
[201] G R Cowgill *The Vitamin B Requirement of Man* Yale University Press 1934
[202] W J Robbins *Science* 89 303 (1939)

and those which are able to synthesize it Among the lower plants and microorganisms which have the power of synthesizing vitamin $B_1$ are yeasts, bacteria, fungi etc Special examples are *Aspergillus niger Agaricus campestris Absidia glauca Bacterium coli, Bacillus pyocyaneus, Chlamydomonas Chlorogonium* and *Polytoma obtusum* (see also page 101) Since many of these organisms contain no chlorophyll it must be concluded that chlorophyll is not an essential factor for the synthesis of the vitamin It seems significant however, that plants (peas) raised in the dark contain very little thiamin, whereas the content of this compound in plants increases rapidly in light [203] All higher plants are able to synthesize thiamin It has been demonstrated, for example for tomatoes that the vitamin is synthesized in the shoots In leaves the concentration of vitamin $B_1$ amounts to a constant value of about 25 International Units per 100 g regardless of the botanical family [204] Roots do not have the power of synthesizing vitamin $B_1$ however, they need thiamin for continued cell division in the embryonic region Vitamin $B_1$ is essential for the growth of all species of roots investigated [205] Thus thiamin appears to be a true plant hormone On the other hand tomato roots are able to synthesize the pyrimidine portion of thiamin, but require an external supply of the thiazole part (Experiments done with excised tomato roots ) Pea roots require an external supply of both parts [206] Vitamin $B_1$ is stored in seeds and more specifically in the outer integuments

Some plant species (for example cosmos camellia, etc ) are said to grow more luxuriantly when the roots obtain besides the self synthesized vitamin $B_1$ some of this vitamin from an outside source Under very carefully conducted experiments it has not however been possible to detect any beneficial effects from added vitamin $B_1$ when plants were raised from their seeds [207] It has been shown that in cuttings and after transplanting vitamin $B_1$ promotes root growth [208]

Higher plants not only synthesize vitamin $B_1$ but excrete it through the roots Therefore the soil in the immediate vicinity of plant roots supports a much higher microbial population than that existing outside the plant s

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[203] J Bonner and J Greene *Botan Gaz* 100 226 (1938)

[204] M Pyke *Biochem J* 34 330 (1940)

[205] J Bonner *Am Chem Soc Div Ag Food Chem Meeti g* Sept 1939 Abst 13 F W Went J Bonner and G C Warner *Science* 87 170 (1938)

[206] F Kögl and A J Haagen Smit *Z physiol Chem* 243 209 (1936) J Bonner *Science* 85 183 (1937) W J Robbins and M A. Bartley *Ibid* 85 246 (1937) W J Robbins M A Bartley A ( H gan and L R Richardson *Proc Natl Acad Sc U S* 23 388 (1937)

[207] D I Arnon *Science* 92 64 (1940)

[208] J Bonner *A Chem Soc D Agr Food Chem Meeting* Sept. 1939 Abst 13 F W Went J Bonner and G L. Warner *Science* 87 1 0 (1938)

zone of influence[209] A great number of bacteria yeasts and fungi[210] are known to require an external supply of thiamin for example, *Staphylococcus aureus*,[211] *Phycomyces blakesleanus*[212] and *Polytomella caeca*[213] Most of them are parasites, some are saprophytes Some organisms which need an external supply of thiamin are able to synthesize it from the thiazole and the pyrimidine intermediate, for example, *Phycomyces blakesleanus* *Phytophthoras*, on the other hand, is unable to do so and resembles a higher animal in this respect

A few organisms are known to be able to synthesize only one part of the thiamin molecule either the pyrimidine part, for example *Polytoma caudatum* and *Chilomonas paramaecium*[214] or the thiazole part These organisms however can combine the portion synthesized with the portion obtained from outside when properly administered

The mechanism of the action of thiamin in plants is not known It is however reasonable to assume that thiamin functions similarly in plants as it does in animals (see page 135)

It should finally be noted that vitamin $B_1$ also promotes alcoholic fermentation by yeast[215] which effect can be used for the determination of the vitamin (see page 131) The growth of acetic acid bacteria is also stimulated by the vitamin[216]

## 15 Animal Physiology

### (a) *Metabolism of Vitamin $B_1$*

Vitamin $B_1$ is completely absorbed in the small gut and partly secreted in the gastric juice probably by means of diffusion[217] It has already been mentioned that vitamin $B_1$ is not stored in the organism, but relatively higher concentrations are found in the liver, kidneys, heart muscles and brain[218] The amount actually present is enough to maintain proper life only for a few days A daily intake of vitamin $B_1$ is, therefore, necessary

209 P M West *Nature* 144 1050 (1939)
210 R J Williams and R R Roehm *J Biol Chem* 87 581 (1930) H Burgeff *Ber deut botan Ges* 52 384 (1934) W H Schopfer *Arch Mikrobiol* 6 510 (1935) F Kögl and N Fries *Z physiol Chem* 249 93 (1937)
211 B C J G Knight *Nature* 139 628 (1937) *J Soc Chem Ind* 56 445 (1937) *Biochem J* 31 731 (1937)
212 W H Schopfer *Compt rend* 200 1965 (1935) *Z Vitaminforsch* 4 187 (1937) W H Schopfer and A Jung *Compt rend* 204 1500 (1937) H M Sinclaire *Nature* 140 361 (1937)
213 A Lwoff and H Dusi *Compt rend* 205 630 (1937)
214 A Lwoff and H Dusi *Ibid* 205 756 (1937)
215 A Schultz L Atkin and C N Frey *J Am Chem Soc* 59 948 (1937)
216 T J Palei *Mikrobiol* 7 843 (1938)
217 M Stockholm T L Althausen and H J Borson *Proc Soc Exptl Biol Med* 46 387 (1941)
218 H G K Westenbrink *Arch neerland physiol* 17 560 (1932) 19 116 (193?)

The organism absorbs only as much vitamin $B_1$ as is needed for the time being All excess is excreted and to a small extent destroyed Even intramuscular injection causes immediate excretion in the urine A vitamin $B_1$ deficiency can be detected from the amount of vitamin excreted in the urine a normal person should excrete 20–80 International Units per day Vitamin $B_1$ is also secreted in milk and eggs

Vitamin $B_1$ is present in the organism both in the free and esterified forms The pyrophosphoric acid ester has been identified as cocarboxylase The presence of vitamin $B_1$ monophosphate in the animal organism is suspected Both vitamin $B_1$ and cocarboxylase occur also in combination with proteins [219]

The vitamin $B_1$ may be absorbed in the intestines in the free or in the phosphorylated form Phosphatase from the duodenum (pigs) can phosphorylate vitamin $B_1$ *in vitro* [220] But it seems that vitamin $B_1$ circulates in blood plasma and in cerebrospinal fluid[221] in the free form (concentration about 1 $\gamma$ per 100 cc ) which diffuses readily and passes into tissue fluid cerebrospinal fluid urine and cells of the body A constant phosphorylation and dephosphorylation take place inside the cells Liver kidney[222] and to a lesser extent, muscle and brain[223] can convert the vitamin into cocarboxylase Nucleated blood cells and probably all nucleated animal cells can phosphorylate vitamin $B_1$ [224] The theory has been advanced that the mammalian non nucleated erythrocytes obtain their cocarboxylase while in the nucleated form within the bone marrow [224]

In blood the cocarboxylase is entirely confined to the blood cells, whereas the free vitamin occurs only in the serum Of the total vitamin $B_1$ in blood nearly 90% is in the phosphorylated form There seems to be a constant level of about 0 5 $\gamma$ of free vitamin $B_1$ per 100 cc of blood

### (b) *Physiological Action of Vitamin $B_1$*

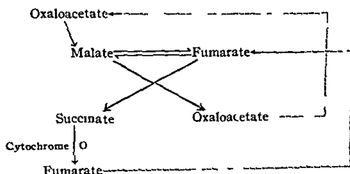
Vitamin $B_1$ in the form of its pyrophosphoric acid ester is intimately concerned with the carbohydrate metabolism Specifically this vitamin is involved in the utilization of pyruvic acid [225] an intermediary degradation product of carbohydrates both in alcoholic fermentation and in tissue me

[219] R. S Goodhart and H M Sinclair *Biochem J* 33 1099 (1939)
[220] H Tauber *Science* 86 180 (1937) *Enzymologia* 2 171 (1937) *J Biol Chem* 123 499 (1938)
[221] H M Sinclair *Biochem J* 33 1816 (1939)
[222] H G K. Westenbrink and J Goudsmit *Enzymologia* 5 307 (1938)
[223] S. Ochoa and R A Peters, *Biochem J* 32 1501 (1938)
[224] R. L. Goodhart and H M Sinclair *Ibid* 33 1099 (1939)
[225] See the review by R A Peters, *Current Science* 5 207 (1936)

can be obtained by the addition of oxaloacetic acid or of pyruvic acid in the presence of carbon dioxide The rate of pyruvate utilization in these experiments depended upon the concentration of carbon dioxide used, thus indicating the formation of oxaloacetic acid according to reaction (2) This pyruvic acid metabolism through the citric acid cycle occurs apparently in many animal tissues, such as liver, brain and several others, in plants, molds and in those types of bacteria[240] in which citrate, succinate, malate or oxaloacetate[241] is formed, but seems to be of minor importance in skeletal[242] and heart muscles[243]

The concept that vitamin $B_1$ is concerned in reaction (2) explains a number of other observations Thus, in contrast to the apparent universal decarboxylation of α oxo carboxylic acids by yeast, animal tissues have been found to utilize pyruvic acid exclusively[244] In *in vitro* experiments with rat kidney slices the synthesis of citric acid from pyruvic acid was found to be markedly accelerated by vitamin $B_1$[245] *In vivo* a deficiency of vitamin $B_1$ in the diet of rats results in decreased excretion of citric acid through the urine,[246] but on the basis of the results obtained from paired feeding experiments it is indicated that the decrease in citric acid secretion is correlated with a diminished intake of food rather than with absence of vitamin $B_1$ *per se*[247]

(*b*) *The Succinic Acid Cycle* The succinic acid cycle[248] may be considered as a variation of the citric acid cycle in that citric acid is not in

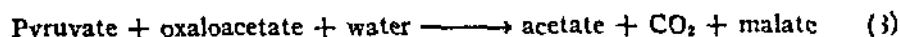



volved and in that there is a difference in the nature of the oxidative and reductive reactions The common principle of both cycles is the reduction

240 H G Wood and C H Werkman *Biochem J* 34 129 (1940)
241 A I Virtanen and T Laine *Nature* 141 748 (1938)
242 H A Krebs and L V Eggleston *Biochem J* 34 442 (1940) H A. Krebs *Ibid* 34 460 (1940)
243 H A Krebs and L V Eggleston *Ibid* 34 1383 (1940)
244 G K McGowan and R A Peters *Ibid* 31 1637 (1937)
245 E S G Barron and C M Lyman *Science* 92 337 (1940)
246 H A. Sober M A Lipton and C A Elvehjem *J Biol Chem* 134 605 (1940)
247 A H Smith and C E Meyer *Ibid* 139 227 (1941)
248 K. Laki F B Straub and A Szent Györgyi *Z physiol Chem* 247 1 (1937)

of one part of the oxaloacetate (or pyruvate) at the expense of the oxidation of another part [249]

According to the succinic acid cycle oxaloacetate is reduced to malate at the expense of an oxidation of pyruvate Thus this reaction may be formulated [49]



$$\text{Pyruvate} + \text{oxaloacetate} + \text{water} \longrightarrow \text{acetate} + CO_2 + \text{malate} \qquad (3)$$

The malate formed according to (3) may react further in two different ways It may react with another molecule of pyruvate (4)

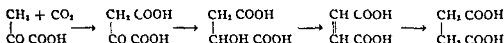


$$\text{Malate} + \text{pyruvate} \longrightarrow \text{oxaloacetate} + \text{lactate} \qquad (4)$$

Or malate may according to the succinic acid cycle be converted into fumarate and finally into succinate

It is postulated that all these reactions occur at considerably different rates in various tissues They serve well to explain the experimental findings The succinic acid cycle appears to be involved in the metabolism of pyruvic acid through reaction (2) in various bacteria and to a certain extent also in animal tissues

In certain bacteria anaerobic fermentation of pyruvate yields [49] malate fumarate and succinate and results in an accumulation of the latter as postulated by the succinic cycle concept This reaction can then be written [250]



$$\begin{matrix} CH_3 + CO_2 \\ | \\ CO\ COOH \end{matrix} \longrightarrow \begin{matrix} CH_2\ COOH \\ | \\ CO\ COOH \end{matrix} \longrightarrow \begin{matrix} CH_2\ COOH \\ | \\ CHOH\ COOH \end{matrix} \longrightarrow \begin{matrix} CH\ COOH \\ \| \\ CH\ COOH \end{matrix} \longrightarrow \begin{matrix} CH_2\ COOH \\ | \\ CH_2\ COOH \end{matrix}$$

and occurs in *Propionibacterium* *B coli* and other species In the case of *B coli* it was shown[251] that the yield of succinate depends upon the pressure of $CO_2$ Succinic acid formation from pyruvic acid has also been demonstrated in certain animal tissues such as for example in the kidney of rats [25] In experiments in which the fixation of radioactive carbon dioxide by bacteria was studied the radioactive element was actually found in the succinic acid isolated from the reaction products[253] and by degradation of the succinic acid it was shown that the radioactive carbon is exclusively in the carboxyl groups of the acid [254]

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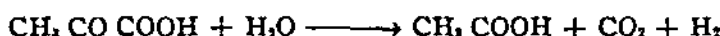
[249] H A. Krebs and L V Eggleston *Biochem J* 34 1383 (1940)
[250] H G Wood and C. H Werkman *Ibid* 34 7 (1940)
[251] S. R Elsden *Ibid* 32 187 (1938)
[252] P S G Barron C. M Lyman M A Lipton and J Goldinger *Proc Am Soc Biol Chem* 1941 XI
[253] H G Wood C. H Werkman A Hemingway and A O Nier *J Biol Chem* 139 265 (1941)
[254] H G Wood C H Werkman A Hemingway and A O Nier *Ibid* 139 277 (1941)

The reaction mechanism in certain other bacteria such as in *Staphylococci*[255] and *Lactobacillus delbruckii* is somewhat different [256, 257] In these organisms the reaction proceeds mainly through reaction (3) and results in an accumulation of acetic acid The other reaction products are totally metabolized

In animal tissues for example in the brain and in certain bacteria, lactic acid is accumulated, [55, 258, 259] which originated from the succinic cycle according to reaction (4) [249]

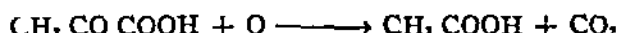
In most animal tissues and in bacteria, all these reactions occur at the same time but at different rates Thus, in quantitative experiments with brain tissue,[260] pyruvic acid was utilized in the following manner 67% was completely metabolized according to the citric acid cycle yielding only $CO_2$, 29 5% was carried through the succinic acid cycle yielding besides $CO_2$, acetic acid and lactic acid

Certain other explanations for the reaction mechanism of the pyruvic acid metabolism in the presence of vitamin $B_1$ have been postulated at various times They all differ from the mechanism as presented in the previous paragraphs in that reaction (2) the initial formation of oxaloacetic acid is not postulated As the result an involvement of the citric and succinic acid cycles was not considered Thus the formation of acetic acid has been explained as decarboxylation (according to reaction (1)) with simultaneous dehydrogenation [261]



$$CH_3\,CO\,COOH + H_2O \longrightarrow CH_3\,COOH + CO_2 + H_2$$

or



$$CH_3\,CO\,COOH + O \longrightarrow CH_3\,COOH + CO_2$$

The occurrence of lactic acid has been explained as a dismutation [262, 263, 264]



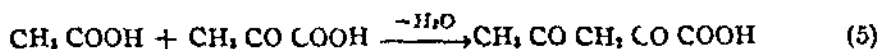
$$2CH_3\,CO\,COOH \rightarrow CH_3\,CHOH\,COOH + CH_3\,COOH + CO_2$$

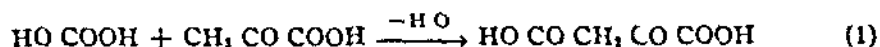
It has been suggested that the dehydrogenations and dismutations are accomplished through some flavin adenine enzyme systems[265] while only the decarboxylations are carried out by the vitamin $B_1$ pyrophosphate enzyme system These explanations do not appear attractive in the light of the previously discussed hypothesis and the evidence for an intermediary formation of oxaloacetic acid by means of the vitamin $B_1$ enzyme system

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255 E S G Barron and C M Lyman *J Biol Chem* 127 143 (1939)
256 F Lipmann *Enzymologia* 4 64 (1937) *Nature* 140 25 (1937) 143 436 (1939)
257 G M Hills *Biochem J* 32 383 (1938)
258 H A Krebs *Nature* 138 288 (1936)
259 H A Krebs *Biochem J* 31 661 (1937)
260 C Long *Ibid* 32 1711 (1938)
261 F Lipmann *Enzymologia* 4 64 (1937) *Nature* 140 25 (1937) 143 436 (1939)
262 H A Krebs *Nature* 138 288 (1936)
263 E S G Barron and C M Lyman *J Biol Chem* 127 143 (1939)
264 H A Krebs *Biochem J* 31 661 (1937)
265 F Lipmann *Nature* 143 281 (1939)

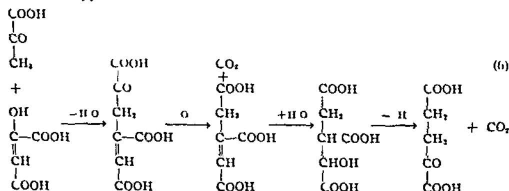
The initial formation of oxaloacetate from pyruvate under the influence of diphospho vitamin $B_1$ also makes a condensation of pyruvic acid with other acids plausible Thus it might be possible [6] that acetic acid condenses with pyruvic acid (reaction (5)) in strict analogy to the condensation of CO with pyruvic acid (reaction (1))




Actually the formation of acetoacetate in pigeon [66] and chicken [67] liver has been demonstrated to be a function of vitamin $B_1$ pyrophosphate and might be explained by an initial formation of acetopyruvate according to reaction (5)

The citric and the succinic acid cycles are probably not the only reactions involved in the utilization of pyruvic and oxaloacetic acids At least one other mechanism must be assumed, since in radioactive α keto glutaric acid obtained in rat liver from pyruvic acid and radioactive sodium bicarbonate the radioactive carbon atom is fixed solely in the carboxyl group which is in α position to the keto group as evident from degradation reactions [26a] Thus this α keto glutaric acid cannot be formed according to the citric acid cycle which would require an equal distribution of the radioactive carbon in both carboxyl groups Another series of reactions (6) has therefore been postulated [68] for the formation of this acid from oxalo acetic and pyruvic acids



So far the action of vitamin $B_1$ in the carbohydrate metabolism has been discussed only in its relation to the utilization of pyruvic acid While this is apparently the most important action other observations may be men

[66] H A. Krebs and L V Eggleston *Biochem J* 34 1383 (1940)
[67] E S G Barron C. M Lyman M A Lipton and J Goldinger *J ... Am Soc Biol Chem* 1941 XI
[68] H G Wood C H Werkman A Hemingway and A. O Nier *J Biol Chem* 139 483 (1941)

tioned concerning a disturbed carbohydrate metabolism during vitamin $B_1$ deficiency Thus, glycogen, the specific animal carbohydrate, requires the presence of this vitamin to be properly metabolized An increase of the glycogen content in liver and in heart muscles has been observed in pigeons during avitaminosis [269] In the urine of children suffering from vitamin $B_1$ deficiency, methyl glyoxal is found [270] In rabbits intramuscular injection of vitamin $B_1$ causes hypoglycemia in amounts of 0 75 mg , while 2 mg or more bring about marked hyperglycemia [271] In man it has generally been observed that an increased carbohydrate metabolism, for example, after an intake of large amounts of carbohydrates during physical labor or during fever periods, requires an increased vitamin $B_1$ utilization

The fat metabolism is influenced by vitamin $B_1$ only in so far as the synthesis of fat from carbohydrates is concerned that is, the metabolism of ingested fats apparently does not need any vitamin $B_1$ On the other hand the utilization of acetic acid as an intermediate in the carbohydrate breakdown appears to be accelerated in bacteria by the presence of vitamin $B_1$ [27] Fat synthesis in experimental animals for example in pigeons and in rats, on a pure carbohydrate diet, is dependent upon the presence of vitamin $B_1$ [273, 274]

The water metabolism in the organism also seems connected with the action of vitamin $B_1$ probably through the carbohydrate metabolism Edema and water imbibitions in the heart and in other organs are symptoms of $B_1$ avitaminosis Vitamin $B_1$ also takes part in the regulation of the nervous system During nerve excitement, two compounds are liberated from the nerve acetylcholine and vitamin $B_1$ [275] It has been shown experimentally that vitamin $B_1$ cannot bring about contractions of the gut, whereas the acetylated vitamin $B_1$ has the power to do so,[276] as does acetylcholine Acetyl thiamin like acetylcholine can be hydrolyzed enzymatically perhaps even by the same acetylcholine esterase [277] It has, therefore been suggested that actually not vitamin $B_1$ but an ester is

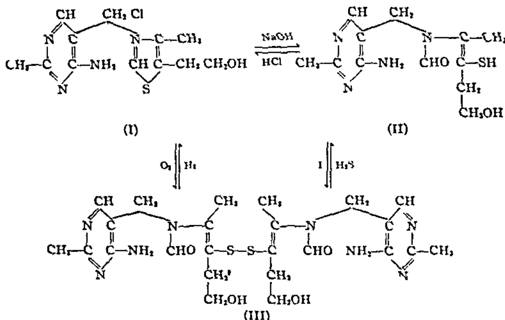
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269 E Abderhalden and W Wertheimer *Arch ges Physiol (Pflügers)* 233 395 (1933)
270 A Geiger and A Rosenberg *Klin Wochschr* 12 1258 (1933)
271 C Ortoleva *Biochim terap sper* 25 511 (1938)
272 J H Quastel and D M Webley *Nature* 144 633 (19-9)
273 E W McHenry *Science* 86 00 (1937) E W McHenry and G Gavin *J Biol Chem* 125 653 (1938) E W McHenry and G Gavin *ibid* 128 45 (1939)
274 E W McHenry *J Physiol* 89 287 (1937) H E Longenecker G Gavin and E W McHenry *J Biol Chem* 134 693 (1940)
275 B Minz and R Agid *Compt rend* 205 576 (1937) L Binet and B Minz *Arch intern physiol* 42 281 (1936) B Minz *Compt rend soc biol* 127 1251 (1938)
276 R Kuhn T Wieland and H Huebschmann *Z physiol Chem* 259 48 (1939)
277 L Massart and R Dufait *Naturwissenschaften* 27 567 (1939) H Sullmann and H Birkhäuser *Schweiz med Wochschr* 69 648 (1939) L Massart and R Dufait *Enzymologia* 7 385 (1939)

liberated from the nerves.[78] It should, however, be noted that one would expect rapid removal of a mediator of nerve impulses from the site of action. Thus, acetylcholine is made inactive hydrolytically by choline esterase. The enzymatic hydrolysis of acetyl thiamin by horse serum and by brain extracts proceeds, however, very slowly.[79]

### (c) *Mechanism of the Vitamin $B_1$ Action*

Vitamin $B_1$ takes part in tissue oxidations of carbohydrates. It might, therefore, be expected that vitamin $B_1$ acts as a compound capable of reversible oxidation and reduction. This is apparently the case. Vitamin $B_1$ (I) represents the reduced form and can be oxidized to a disulfide (III).[80] This oxidation occurs under physiological conditions, for example,

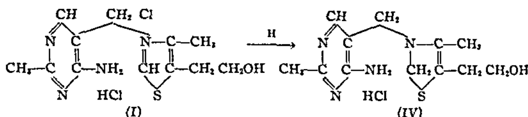



ample, at pH 7.5 with hydrogen peroxide or oxygen from air,[231] but can also be brought about by oxidation in alkaline solution with iodine. On the other hand, the disulfide can be reduced to the thiol form (II) of vitamin $B_1$ by hydrogen, hydrogen sulfide, glutathione or cysteine,[81] and the thiol form is converted into the vitamin by acids such as hydrochloric acid. In

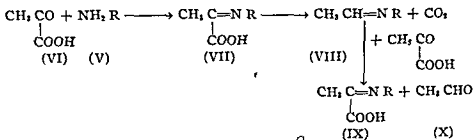
[78] H. U. Graf and A. v. Muralt, *Angew. Chem.*, 52, 463 (1939).
[79] H. Süllmann and H. Birkhäuser, *Schweiz. med. Wochsch.*, 69, 648 (1939). L. Massart and R. Dufait, *Naturwissenschaften*, 27, 567 (1939). D. Glick and W. Antopol, *Proc. Soc. Exptl. Biol. Med.*, 42, 396 (1939).
[80] O. Zima and R. R. Williams, *Ber.*, 73, 941 (1940).
[81] O. Zima, K. Ritsert and T. Moll, *Z. physiol. Chem.*, 267, 210 (1941).

body fluids and in tissues, the reduction apparently does not stop at the thiol stage The disulfide shows the full biological activity of the vitamin itself and is about five times less toxic than the vitamin

The possibility that vitamin $B_1$ represents not the reduced but the oxidized form has also been investigated Actually by mild reducing agents for example by the action of hydrosulfite in sodium carbonate solution one atom of hydrogen is absorbed[281] (I → IV) The reduced form is a reducing agent but is non autoxidizable It is also devoid of vitamin activity Furthermore upon pyrophosphorylation the molecule becomes more resistant to the action of reducing agents[282] The possibility therefore that vitamin $B_1$ acts according to this scheme can be discarded




It has also been suggested that vitamin $B_1$ may act through its amino group according to the Langenbeck cycle[284] In this system all primary amines (V) which have been investigated decarboxylate α keto-carboxylic acids (VI) to the corresponding lower aldehyde through the formation of an imino acid (VII) which decomposes into carbon dioxide and an aldimine (VIII) The latter reacts with another molecule of the keto acid thus yielding again one mol of imino acid (IX) and one mol of aldehyde (X) This reaction mechanism is however very improbable since in the actual test system for the Langenbeck cycle vitamin $B_1$ was found to be completely inactive[285]




## (d) *Relation of Vitamin B1 to Other Vitamins, to Hormones, and Minerals*

The close relation of vitamin $B_1$ to the carbohydrate metabolism becomes apparent from the relation of the vitamin to the hormone of the thyroid gland, thyroxine Thyroxine secretion increases the intensity of the general metabolism and hence necessitates an increased amount of vita

[281] F Lipmann *Nature* 138 1097 (1937)
[282] E S G Barron and C M Lyman *Science* 92 337 (1940)
[284] *Ergeb Enzymforsch* 2 314 (1933)
[285] K G Stern and J I Melnick *J Biol Chem* 131 597 (1939)

min $B_1$ [286, 287, 288, 289, 290] A similar relationship can be demonstrated for vitamin $B_1$ and insulin Vitamin $B_1$ increased the sugar tolerance of rats in insulin hypoglucemia experiments [291]

A close relationship exists between one of the hormones of the adrenal cortex and vitamin $B_1$ During vitamin $B_1$ avitaminosis a hypertrophy of the adrenal cortex occurs Since one of the hormones of the adrenal cor tex regulates the lipoid content of blood hypertrophy of the adrenal gland causes increase of the cholesterol content of blood If during $B_1$ avitamino sis extracts of the adrenal cortex are injected hypertrophy of the gland does not occur and the cholesterol content of the blood does not increase Hypertrophy of the gland on the other hand, can apparently be cured by vitamin $B_1$ intake

It has been reported that substances of the cyclo pentano perhydro phenanthrene type, for example, female and male sex hormones and vita mins D [295] delay symptoms of vitamin $B_1$ deficiency These results how ever need confirmation

The relation of vitamin $B_1$ to the other vitamins also needs further study Vitamin A is said to act antagonistically, since increase of the vitamin A intake increases the symptoms of vitamin $B_1$ deficiency On the other hand vitamin A deficiency in rats on a diet containing normal amounts of $B_1$ causes the appearance of symptoms of a $B_1$ deficiency (pyruvic acid in the blood) which can be cured by increased $B_1$ intake [294]

Interesting is the relationship of vitamin $B_1$ to the zinc metabolism During avitaminosis the zinc contents of the blood,[292] the toenails, finger nails and skin are reduced to half their normal values [293] In natural food stuffs there seems to be a correlation of the amount of zinc and vitamin $B_1$ [293]

A possible relation of vitamin $B_1$ to manganese has been observed When rats were fed 50 International Units per day there resulted, after one gen eration interference with lactation loss of maternal instinct cannibalism and progressive loss of fertility When small amounts of manganese chloride (2 mg per day) were added to the diet all these effects disap

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[286] H E Himwich W Goldfarb and C R. Cowgill *Am J Physiol* 99 689 (1938)
[287] G R Cowgill and M L. Palmieri *Ibid* 105 146 (1933)
[288] B Sure and K S Buchanan *J Nutrition* 13 513 (1937)
[289] V A Drill and C R. Sherwood *Am J Phys ol* 124 683 (1938)
[290] R A Peters and R J Rossiter *Biochem J* 33 1140 (1939)
[291] J C B rke and A. R. McIntyre *J Pharmacol* 64 465 (1938)
[292] W G E Eggl ton *Chinese J Physiol* 15 33 (1940)
[293] W G E Eggleton *Biochem J* 33 403 1939)
[294] H v Euler and B Högberg *Naturwissenschaften* 27 769 (1939)
[295] J Sanchez Rodriguez and J M Sarda *Z Vitam nforsch* 6 193 (193")

peared [296] It has also been noted[297, 298] that $Mn^{++}$ greatly stimulates the carboxylase system if present in appropriate concentrations

## 16 Avitaminosis and Hypovitaminosis

Severe cases of vitamin $B_1$ deficiency in man have been and still are very common in the tropics, especially in the Philippines and India and in Japan and are due to poor nourishment, the food consisting mainly of polished rice In America and Europe, severe cases are only occasionally observed and are caused, for example, by chronic alcoholic addiction, by pregnancy, by toxic agents such as nicotine, lead, thallium, arsenic and mercury, etc Cases of more or less severe hypovitaminosis are quite common in the Western countries due to inadequate nutrition

The symptoms generally ascribed to vitamin $B_1$ deficiency are mostly symptoms of combined deficiencies of several vitamins of the B group, especially $B_2$, nicotinic amide, $B_6$ etc Vitamin $B_1$ deficiency in man affects first of all the emotions and the tonus of the nervous system Further symptoms are loss of appetite (anorexia), unusual susceptibility to fatigue combined with lower physical endurance, gastrointestinal disturbances muscular weakness pains and paraesthesia in arms and legs, edema in ankles and face and decrease of the blood pressure In more severe cases the entire nervous system is affected and symptoms of polyneuritis and neuralgia occur The specific polyneuritis due to vitamin $B_1$ deficiency is bilateral, symmetrical and involves predominantly the lower extremities [299] Muscle cramps of the calf are often noted and, at later stages, calf muscle atrophy The position sense in the toes is disturbed and foot drop is manifested The typical form of beriberi, the severe disease of the tropics, is accompanied by symptoms such as lameness ataxia disturbance of the motor and sensory nerves followed by labored breathing and hypertrophy of the right heart and finally death from heart failure Cardiovascular dysfunction may also arise from mild but continued deficiency of vitamin $B_1$ [300]

The symptoms of vitamin $B_1$ deficiency in rats resemble very closely the symptoms of human beings In addition to these, there is a marked influence upon the growth of young animals More severe avitaminosis causes convulsions and paralysis of the lower extremities

[296] D Perla *Proc Soc Exptl Biol Med* 37 169 (1937) *Science* 89 132 (1939)
[297] K. Lohmann and P Schuster *Biochem Z* 294 188 (1937)
[298] S Ochoa and R A Peters *Biochem J* 32 1501 (1938)
[299] N Jolliffe *Minnesota Med* 23 542 (1940)
[300] S Weiss and R W Wilkins *Tr A Am Physicians* 51 341 (1936) *Ann Internal Med* 11 104 (1937) W A Jones and B Sure *J Lab Clin Med* 22 991 (1937)

The symptoms in pigeons which have been used for the biological detection of the vitamin are quite characteristic. These birds first show lassitude and eat little. They sit with ruffled feathers and draw their heads far back and upside down (head retraction, opisthotonus). Convulsions may follow. Two or three days after these symptoms appear the bird dies.

### (a) *Clinical Test Methods*

A diagnosis for vitamin $B_1$ deficiency can be carried out by a number of different procedures. The amount of this vitamin present in blood and in urine can be determined. The cocarboxylase content in blood can also be used as an assay procedure. Finally, a determination of the amount of pyruvic acid present in blood or in urine can serve as an indication for a vitamin $B_1$ deficiency. In addition to these direct methods determinations of the tissue saturation with vitamin $B_1$ may be carried out in which the effects of defined doses of vitamin $B_1$ are measured by any of the indicated methods before and after administration.

**Urine Tests.** The urinary output of vitamin $B_1$ is closely related to the state of nutrition of the body. Healthy humans on an adequate diet excrete between 50 and 150 $\gamma$ vitamin $B_1$ per day.[301, 302, 303, 304] Lower values are found during vitamin $B_1$ deficiency,[305] pregnancy[304] and a variety of diseases.[303, 306] Vitamin $B_1$ pyrophosphate (cocarboxylase) does not appear normally in the urine.

For the actual *determination of vitamin $B_1$* a number of different methods have been recommended. The *bradycardia assay*[307] for example has given most satisfactory results. The *thiochrome method* must be modified since urine contains fluorescent compounds which interfere with the thiochrome determination. Various modifications of the basic method have been recommended[306, 308, 309, 310, 311] and have been applied successfully as

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301 J Houston S K Kon and S Y Thompson *J Soc Chem Ind* 58, 651 (1939) J Houston and S. K. Kon *Nature* 143 558 (1939)

302 K. Rassett *Klin Wochschr* 17 1237 (1938)

303 H Schroeder *Ibid* 18 148 (1939)

304 H G K Westenbrink and J Goudsmit *Arch néerland Physiol* 23 ?9 (1938)

305 L J Harris P C Leong and C C Ungley *Lancet* 234 539 (1938) L J Harris and P C. Leong *Ibid* 1 886 (1936)

306 Y L. Wang and L J Harris *Biochem J* 33 1356 (1939) Y L Wang and J Yudkin *Ibid* 34 343 (1940)

307 T W Birch and L. J Harris *Ibid* 28 60? (1934)

308 J Marrack and H F Höllering *Lancet* 236 325 (1939)

309 G M Hills *Biochem J* 33 1966 (1939)

310 H G K Westenbrink J Goudsmit and B C P Jansen *Nature* 139 1108 (1937) *Rec trav chim* 56 803 (1937) *Acta Brev Neerland Physiol Pharmacol Microbiol* 8 ?1 119 (1938)

311 M Jowett *J Soc Chem Ind* 58 556 (1939) *Biochem J* 34 1348 (1940)

clinical test methods especially when larger quantities of the vitamin are being assayed such as in saturation tests The *colorimetric method by Prebluda and McCollum* has also been applied successfully to the assay of vitamin $B_1$ in urine [312] The *yeast fermentation method* offers promising results for urine tests [313, 314] Since urine contains, however other substances which stimulate the fermentation, it has been recommended[315] to carry out a determination before and after an oxidative inactivation of the vitamin In a parallel test in which vitamin $B_1$ has been added to the urine the efficiency of the inactivation procedure is evaluated

*Pyruvic acid determinations*[316, 317, 318, 319, 320, 321, 322, 323, 324, 325] can be made to detect a state of vitamin $B_1$ deficiency One assay procedure is based on the isolation of sodium pyruvic acid 2,4 dinitrobenzoate and the red color developed by the latter with strong alkali In practice, the urine (or the blood) is mixed with trichloroacetic acid (or in the case of blood, better with tungstic acid) and a solution of 2,4 dinitro phenyl hydrazine in diluted hydrochloric acid solution is added The hydrazones formed are extracted with ethyl acetate followed by an extraction with a sodium carbonate solution Finally a sodium hydroxide solution is added and the color developed is measured This method is of course, not specific for the determination of pyruvic acid since other $\alpha$ keto carboxylic acids such as acetoacetic acid, oxaloacetic acid and $\alpha$ keto glutaric acid, give the same reaction Pyruvic acid can also be estimated by determination of bisulfite binding substances According to this procedure urine is mixed with solutions of oxalic acid and of sodium bisulfite After an appropriate length of time, the excess of bisulfite is removed and the bisulfite bound substances are titrated iodometrically

The amount of pyruvic acid in the urine of the normal and vitamin $B_1$ deficient man has not been studied systematically In rats the amount excreted increases during vitamin $B_1$ deficiency 200–400% but is also a

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[312] D Melnick and H Field *J Biol Chem* 123 83 (1938) 130 97 (1939) *Proc Soc Exptl Biol Med* 38 723 (1938)
[313] A S Schultz L Atkin and C N Frey *J Am Chem Soc* 59 948 2457 (1937) 60 1514 (1938)
[314] A S Schultz L Atkin and C N Frey *Proc Soc Exptl Biol Med* 38 404 (1938)
[315] A S Schultz L Atkin and C N Frey *J Biol Chem* 136 713 (1940)
[316] B S Platt and G D Lu *Quart J Med* 5 355 (1936) G D Lu *Biochem J* 33 249 (1939)
[317] F P Clift and R R Cook *Biochem J* 26 1788 (1932)
[318] G G Banerji and L J Harris *Ibid* 33 1346 (1939)
[319] M Shils H G Day and E V McCollum *Science* 91 341 (1940)
[320] H A Harper and H J Deuel *J Biol Chem* 137 233 (1941)
[321] D Klein *Ibid* 137 311 (1941)
[322] E M Case *Biochem J* 26 753 (193?)
[323] R A Peters and R H S Thompson *Ibid* 28 916 (1934)
[324] E Bueding and H Wortis *J Biol Chem* 133 585 (1940)
[325] M Shils H G Day and E V McCollum *Ibid* 139 145 (1941)

function of the type and quantity of food taken in The urinary output of pyruvic acid appears to be higher in the male than in the female

**Blood Tests** The *determination of vitamin $B_1$ in plasma* is rather difficult since the concentration is normally very small, about 1 $\gamma$ in 100 ml Furthermore slight hemolysis of the blood affects the results considerably[326] The only method therefore which has been found useful is the determination of the growth rate of *Phycomyces blakesleanus*[327] Blood contains however, other factors which increase the growth rate of this fungus Nevertheless the method is valuable for comparing the apparent vitamin $B_1$ content in different samples of blood[328] The difficulties can partly be overcome by evaluation of the growth rate by blood with and without the addition of vitamin $B_1$

The *determination of vitamin $B_1$ pyrophosphate (cocarboxylase)*[326, 329, 330, 331] can be carried out much more easily The pyrophosphate occurs exclusively in the blood cells Therefore in cases in which the blood cell count is disturbed for example in cases of polycythemia or of myeloid leukemia the cocarboxylase determination should not be used The method consists in the determination of the carbon dioxide production from pyruvic acid in the presence of yeast as a source of carboxylase and in the presence of excess amounts of vitamin $B_1$ Instead of using whole blood for the determination the use of washed blood cells has been recommended Blood of healthy adults contains an average of about 7 $\gamma$ of cocarboxylase per 100 ml A value below 3 $\gamma$ is considered as indicative of a state of vitamin $B_1$ deficiency

The *determination of pyruvic acid* is carried out as described for its determination in urine A marked increase in pyruvic acid has been found in man and in experimental animals, such as in pigeons[33] and in rats[333] during times of vitamin $B_1$ deficiency The normal pyruvic acid content of human blood averages 2 8 mg per 100 cc

## 17 Hypervitaminosis

Vitamin $B_1$ has no specific pharmacological action as far as is known to day The vitamin is non toxic even in doses which are several thousand times larger than a normal daily dose No cases of human vitamin $B_1$

[326] R Goodbart and H M Sinclair *Biochem J* 33 1099 (1939)
[327] H M Sinclair *Ibid* 33 2027 (1939)
[328] H M Sinclair *Ibid* 32 2185 (1938)
[329] S. Ochoa and R. A Peters *Ibid* 32 1501 (1938)
[330] R. Goodbart and H M Sinclair *J Biol. Chem* 132 11 (1940)
[331] R. Goodhart *Ibid* 135 77 (1940)
[332] R. H S. Thompson and R E Johnson *Biochem J* 29 694 (193 )
[333] C D Yu *Ibid.*, 33 774 (1939)

hypervitaminosis have ever been reported The therapeutic index, that is, the ratio of the therapeutic dose to the minimum lethal dose, is extremely high 600 for mice, 5000 for rats and 70,000 for dogs Doses of 125 mg per kg body weight for mice, 250 mg per kg for rats, 300 mg per kg for rabbits, or 350 mg per kg for dogs are lethal when injected intravenously [334]

## 18 Requirements

It is impossible to define accurately the requirements of vitamin $B_1$ The amount needed is not a constant, but a function of the supplied food of the intensity of the metabolism, of the outside temperature and of other factors The consumption of carbohydrates increases and of fat decreases the amount of vitamin $B_1$ needed by the organism, while proteins do not have any influence on the vitamin requirement The intensity of the total metabolism is again a function of many single factors, among which might be mentioned the secretion of thyroxin, the physical labor performed, pregnancy, etc

The ratio of the minimum amount of vitamin $B_1$ needed to support life to the optimal amount for general development of the body is very high and can vary from 3 up to 100

*The vitamin $B_1$ requirement of man therefore cannot be defined exactly* The allowances as recommended by the Food and Nutrition Board of the National Research Council (for details see page 613) call for a daily intake varying from 1 2–2 3 mg of thiamin for adults depending upon sex and relative activity The requirements for children are correspondingly lower A six to eight months old baby should receive 0 4 mg of thiamin [335]

Vitamin $B_1$ is as far as is known needed by all animals even by insects and microorganisms *Sheep*[336] *and cattle*[337, 338, 339] *apparently need no external supply of this vitamin, since it is synthesized by bacteria in the rumen of these animals*

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[334] H Molitor *Merck s Jahresberichte* 1936 51
[335] E M Knott *Proc Soc Exptl Biol Med* 45 765 (1940)
[336] L W McElroy and H Goss *J Biol Chem* 130 437 (1939)
[337] L W McElroy and H Goss *Ibid* 133 LXV (1940)
[338] M I Wegner A N Booth C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 45 769 (1940)
[339] S I Bechdel H E Honeywell R. A Dutcher and M H Knutsen *J Biol Chem.*, 80, 231 (1928)

# VITAMIN $B_2$—
# RIBOFLAVIN

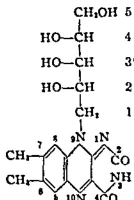
# VITAMIN $B_2$—RIBOFLAVIN

## 1 Nomenclature and Survey

*Names*

Riboflavin name used in America [1]
Lactoflavin name used in Europe
Ovoflavin and hepatoflavin historical names indicating the origin
*Vitamin G* historical designation by Sherman

**Chemical formula**



**Chemical name**

6 7 Dimethyl 9 (*d* 1′ ribityl )iso-alloxazine
6 7 Dimethyl 9-*d* riboflavin

**Empirical formula**



$C_{17}H_{20}N_4O_6$

**Efficacy**

1 g riboflavin = 400 000 Bourquin-Sherman Units

**Classification**

Vitamin $B_2$ belongs to the class of colored water soluble naturally occurring substances which carry the group name lyochromes In distinction from the lyochromes the colored fat or organic solvent soluble naturally occurring substances

[1] The term *d* ribofl n or just ribofl vin was sugg sted by the Cou cil on Pharmacy and Chem try *J Am M d Assoc* 108 1340 (1937) and approved by the Committee on Vitam n Stand rd American Society of Biological Chem t and al o by the Comm ttee on Vit m n Nomencl ture American In ti tute of N trit on

are called *lipochromes* The lyochromes are not as yet systematically subclassified Arbitrarily vitamin $B_2$ is a member of the subdivision flavins The members of this group carry names indicating the origin of their occurrence Thus lactoflavin comes from milk ovoflavin from eggs hepatoflavin from liver uroflavin from urine etc All naturally occurring flavins which have been investigated with the exception of uroflavin proved to be identical with riboflavin

## 2 Chronology

1879 BLYTH[2] isolated an impure flavin from whey

1913 OSBORNE and MENDEL recognized in milk the presence of a water soluble substance which promotes growth [3]

1917 EMMETT and MCKIM differentiated the physiological action of vitamin $B_1$ from vitamin $B_2$ [4]

1925 BLEYER and KALLMANN obtained a yellow pigment called lactochrome in crude form from milk [5]

1932 WARBURG and CHRISTIAN isolated the yellow enzyme from yeast [6] BANGA and SZENT GYORGYI[7] recognized a respiration co ferment in yeast the colored part of which was called cytoflav

1933 ELLINGER and KOSCHARA and KUHN GYORGYI and WAGNER JAUREGG isolated pure riboflavin The latter authors recognized the identity of vitamin $B_2$ and riboflavin

1935 KUHN and KARRER and their groups established the constitution of riboflavin by total synthesis

## 3 Occurrence

Riboflavin is very widely distributed over the entire animal and plant kingdom [8] It seems that each animal and plant cell contains small amounts The amount in plant seeds is small but increases rapidly during germination The richest source of riboflavin is anaerobic growing fermentation bacteria for example butyric acid bacteria in the dried state contain up to 15 mg % Also various yeasts contain fair amounts of riboflavin Liver kidney and heart of vertebrata and fish livers contain about 10–30 mg % or about 10–30 times more than muscles The retina

[2] A W Blyth *J Chem Soc* 35 530 (1879)

[3] T B Osborne and L B Mendel *J Biol Chem* 15 311 (1913)

[4] A D Emmett and L H McKim *Ibid* 32 409 (1917)

[5] B Bleyer and O Kallmann *Biochem Z* 155 54 (1925)

[6] O Warburg and W Christian *Naturwissenschaften* 20 688 980 ( ... *Biochem Z* 254 438 (1932) 257 492 (1933) 266 377 (19 ...

[7] I Banga and A Szent Györgyi ... 203 (1932) ... Szent Gyorgyi and I Vargha *Z physiol Chem* 210 2 ...

[8] P Gyorgy R Kuhn and T W ... *physiol Chem* ... H v Euler and J Adler *Ibid* 223 105 (1934)

of the eyes of many species of animals contains considerable quantities of riboflavin [9]

Vitamin $B_2$ occurs in the animal organism in a number of different forms In milk in urine[10] and in the retina,[11] or, generally speaking, in places where no respiration or fermentation takes place the free riboflavin is found In tissues riboflavin occurs as such as riboflavin phosphoric acid and in the form of riboflavin phosphoric acid adenine dinucleotide Each of these forms occurs in the free state as well as combined with specific proteins (see page 171) For a combination of riboflavin with protein in muscles, see[12]

## 4 Isolation

The isolation of riboflavin is carried out by extraction of flavin containing materials with aqueous acid solutions with water alcohol mixtures with alcohol or with acetone Direct extraction of natural materials especially of plant origin yields only part of the total riboflavin present The remaining vitamin is chemically bound but can be liberated by heating [13] The extraction is followed by a combination of different precipitation and adsorption procedures The following precipitation methods have been used [14, 15] lead acetate phosphotungstic acid in normal sulfuric acid followed by extraction of the precipitate with amyl alcohol silver nitrate or mercuric sulfate in acid solution precipitates other substances leaving the vitamin $B_2$ in solution but silver nitrate in neutral solution precipitates the vitamin By the addition of alcohol to concentrated aqueous riboflavin solutions salts and glycogen are eliminated

Riboflavin is adsorbed in acid solution by fuller s earth[16] or in neutral solution by frankonit Charcoal can also be used successfully Talc aluminum oxide calcium carbonate kaolin and kieselguhr do not absorb vitamin $B_2$ The elution is carried out by basic solvents such as pyridine

[9] G Brunner and F Baroni Monatsh 68 264 (1936)

[10] A Emmerie Acta Brevia Neerland Physiol Pharmacol Microbiol 8 116 (1938)

[11] H v Euler and E Adler Ark Kemi Mineral Geol B11 No 28 (1934) R Kuhn and H Kaltschmitt Ber 68 386 (1935) E Adler and H v Euler Nature 141 790 (1938) H v Euler and E Adler Z physiol Chem 223 105 (1934)

[12] J Schormüller Z Untersuch Lebensm 77 1 (1939)

[13] F M Lantz Agr Exptl Sta New Mexico Coll Agr Mech Arts Bull 268 (1939)

[14] B C Guha Biochem J 25 945 (1931)

[15] P György R Kuhn and T Wagner Jauregg Z physiol Chem 223 21 27 236 241 (1934)

[16] A Seidell U S Pub Health Service Pub Health Repts 31 464 (1916) W D Salmon N B Guerrant and I M Hays J Biol Chem 80 91 (1928) B T Narayanan and J C Drummond Biochem J 24 19 (1930)

methanol water mixtures,[17, 18] ammonia, caustic solutions, triethanolamine or by neutral organic solvents, such as 80% acetone After precipitation, together with lead sulfide, riboflavin is extracted with hot water[17, 19]

From concentrates obtained from the elution of adsorbates, riboflavin usually crystallizes out in clusters of fine orange yellow needles They can be purified further by precipitation with thallium ions or by one of the previously described precipitation methods Final recrystallizations are carried out from water, aqueous alcohol or diluted acetic acid

By such methods, the pure vitamin $B_2$ has been obtained by many workers,[20-29] and the identity of the flavins from different sources has been established chemically and biologically Ovoflavin from egg white, lacto flavin from milk, hepatoflavin from liver, etc, are identical with riboflavin One gram of riboflavin was obtained from 5400 liters of milk

Methods for the separation of vitamin $B_1$ from vitamin $B_2$ are discussed on page 102 It might be added here that vitamin $B_1$ can be destroyed by heating to 120° C for six hours, leaving the vitamin $B_2$ mainly intact[30]

For methods of separating riboflavin, riboflavin phosphoric acid and the flavin enzymes from each other see page 183

## 5 Properties

Riboflavin crystallizes in fine orange yellow needles which melt at 282° C under decomposition (darkening at about 240°) The pure compound is slightly soluble in water (12 mg in 100 cc at 27 5°, 19 mg at 40°) and in ethyl alcohol (4 5 mg at 27 5°) amyl alcohol, cyclo hexanol, phenol, amyl acetate, etc , and is very soluble in alkali solutions It is insoluble in acetone, ether, benzene and chloroform The impure material is much

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[17] P Ellinger and W Koshara *Ber* 66 315 808 1411 (1933)

[18] R Kuhn P György and T Wagner Jauregg *Ibid* 66 317 576 1034 1577 (1933) *Naturwissenschaften* 21 560 (1933) *Klin Wochschr* 12 1241 (1933)

[19] P Karrer H Salomon and K Schöpp *Helv Chim Acta* 17 419 735 (1934)

[20] P Ellinger and W Koschara *Ber* 66 315 808 1411 (1933)

[21] R Kuhn P György and T Wagner Jauregg *Ibid* 66 317 576 1034 1577 (1933) *Naturwissenschaften* 21 560 (1933) *Klin Wochschr* 12 1241 (1933)

[22] P Karrer H Salomon and K Schöpp *Helv Chim Acta* 17 419 735 (1934)

[23] L E Booher *J Biol Chem* 102 39 (1933) 107 591 (1934)

[24] S Lepkovsky W Popper and H M Evans *Ibid* 108 257 (1935) 109 Proc 54 (193 )

[25] S Itter E R Orent and E V McCollum *Ibid* 108 579 (1935)

[26] C A Elvehjem and C J Koehn *Ibid* 108 709 (1935)

[27] F J Stare *Ibid* 111 567 (1935)

[28] S Ansbacher G C Supplee and R C Bender *J Nutrition* 11 401 (1936)

[29] R D Greene and A Black *J Am Chem Soc* 59 1820 (1937)

[30] M I Smith and L G Hendrick *U S Pub Health Service Pub Health Repts* 41 201 (1926) A Seidell *Bull soc chim biol* 8 746 (1926) A Hassan and J C Drummond *Biochem J* 21 653 (1927) H Chick and M H Roscoe *Ibid* 21 698 (1927) H C Sherman and J H Axtmayer *J Biol Chem* 75 207 (1927)

more soluble than the pure material The water solution is of greenish yellow color and displays an intense yellow green fluorescence which van ishes on the addition of acids Alkali also causes disappearance of the fluorescence by shifting the hydrogen from the imino group in 3 position to the neighboring oxo group, thus forming an enol The fluorescence (maximum at 565 mμ) is used for the quantitative determination of riboflavin (see page 182) Optimum fluorescence occurs at pH 3 to 9 The isoelectric point of vitamin $B_2$ is at pH 6 Riboflavin is thus

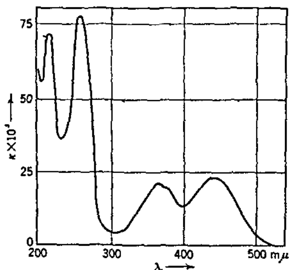



Fig 10 —Absorption spectrum of vitamin $B_2$ (ribo flavin) (R Kuhn)

amphoteric in character The isoelectric constants for acid and base are calculated to be [21] $K_{acid} = 6\ 3 \times 10^{-12}$ and $K_{base} = 0\ 5 \times 10^{-5}$ Ribo flavin shows optical rotation $[\alpha]_D^{20} = -114°$ in 0 1 normal sodium hydroxide [22] In neutral and in acid solution, the optical activity is ex ceedingly small Riboflavin has a characteristic absorption spectrum with maxima at 445 372, 269 and 225 mμ (Fig 10)

Crystalline riboflavin, when protected against light is stable at ordinary temperatures Light slowly destroys the vitamin activity In solution vitamin $B_2$ is essentially unstable The decomposition is greatly in fluenced by light temperature and pH of the solution Under alkaline

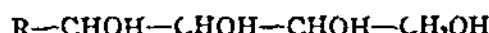
[21] R Kuhn and G Moruzzi *Ber* 67 888 (1934)

[22] R Kuhn and H Rudy *Ibid* 68 169 (1935) P Karrer and H Fritzsche *Helv Chim Acta* 18 … (1935)

conditions riboflavin decomposes rapidly Riboflavin possesses a relatively high degree of thermostability thus only slight destruction occurs by heating to 120° C for six hours

### 6 Chemical Constitution of Vitamin $B_2$ Degradation Reactions

Riboflavin has the empirical formula $C_{17}H_{20}N_4O_6$ It is resistant against acids, bromine and oxidizing agents such as hydrogen peroxide and concentrated nitric acid Chromic acid oxidizes the molecule to ammonia, carbon dioxide and a nitrogen free residue of unknown constitution Acetylation of riboflavin yields a tetra acetate, indicating the presence of four hydroxyl groups [33, 34] A diacetone compound can be formed, which indicates the close position of each two hydroxyl groups [35] *Oxidation* of riboflavin with lead tetra acetate yields 0 8 mol of formaldehyde A primary hydroxyl group is, therefore, in $\alpha$ position to a secondary hydroxyl group [34] Primary amino groups are not present since nitrous acid does not affect riboflavin Alkaline hydrolysis yields urea [33] indicating the presence of the configuration —NH—CO—NH— The other two nitrogen atoms are tertiary Irradiation destroys vitamin $B_2$ Irradiation in alkaline solution yields a new compound, called lumiflavin (lumi lacto flavin) or photoflavin and a sugar compound $C_4H_8O_4$, which could not be isolated as such, probably because of decomposition Lumiflavin cannot be acetylated further and does not give formaldehyde by oxidation with lead tetra acetate Vitamin $B_2$, therefore, contains a side chain of the constitution of a tetrahydroxy butyl group,



$$R—CHOH—CHOH—CHOH—CH_2OH$$

*the configuration of which could only be determined by total synthesis of* riboflavin It proved to be *d* ribose

Photolysis of riboflavin in neutral solution *in vacuo* causes the disappearance of the yellow color with the *formation of deutero leuco riboflavin* which can be dehydrogenated by oxygen Alkali causes the dehydrogenated compound to be converted into the already mentioned lumiflavin [36]

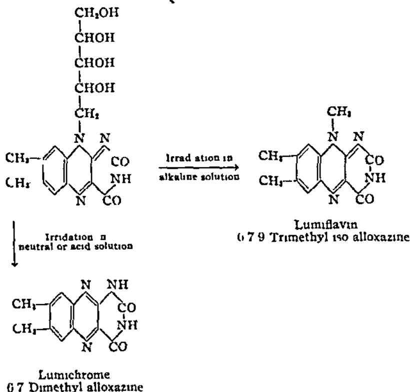
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[33] R Kuhn and T Wagner Jauregg *Ber* 66 1577 (1933)

[34] R Kuhn H Rudy and T Wagner Jauregg *Ibid* 66 1950 (1933)

[35] R Kuhn H Rudy and F Weygand *Ibid* 68 62 (1935)

[36] R Kuhn H Rudy and T Wagner Jauregg *Ibid* 66 1950 (1933)

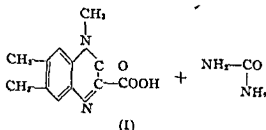


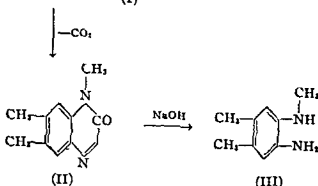

Lumiflavin
6 7 9 Trimethyl iso alloxazine

Lumichrome
6 7 Dimethyl alloxazine

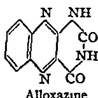
Lumiflavin contains a methyl imid group[37] which was not present in riboflavin and which, therefore, *must have replaced the missing sugar side chain* The hydroxyl groups of the side chain are therefore connected with the flavin ring system by a methylene group Alkaline hydrolysis of lumiflavin yields urea and an oxo carboxylic acid, $C_{12}H_{12}N_2O_3$[38, 39] (I), which contains one active hydrogen atom and exhibits the properties of a monobasic acid upon titration Since two molecules of water are required for the alkaline hydrolysis of lumiflavin the urea must come from a ring *system and not from a side chain ureide or guanidino group which would require only one molecule of water for hydrolysis* The oxo acid (I) is decarboxylated upon heating yielding the lactam (II) By heating the lactam with sodium hydroxide, 1,2 dimethyl-4 amino 5 methyl amino benzene (III) is formed This *o* phenylene-diamine gives a bluish green color reaction with ferric chloride, which is, according to Noelting, characteristic for *p,p*-disubstituted *o* phenylene-diamines

[37] R Kuhn and H Rudy *Ber* 67 1798 (1934)
[38] R Kuhn and T Wagner Jauregg *Ibid* 66 1577 (1933)
[39] R Kuhn and H Rudy *Ibid* 67 892 (1934)

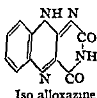



Irradiation of riboflavin yields lumiflavin in alkaline solution and lumichrome, in neutral or in acid solution [40] Lumichrome has the constitution of 6,7-dimethyl alloxazine, and is an intensely fluorescent compound It is a derivative of alloxazine as proved by synthesis, whereas lumiflavin and riboflavin are derivatives of the hypothetical iso alloxazine Lumiflavin and riboflavin have one active hydrogen atom in 3 position lumichrome contains active hydrogens in both 1- and 3 positions



Alloxazine



Iso alloxazine

Riboflavin readily takes up two hydrogen atoms[41] It is reversibly reduced by hydrogen in the presence of a catalyst, by zinc in acid solution, by sodium thiosulfate, by hydrogen sulfide in alkaline solution and by titanous chloride The dihydro compound is colorless, *shows no fluorescence* and is called leuco riboflavin (leuco lactoflavin) The leuco compound is easily oxidized to riboflavin by air The redoxy potential of an equimolecular mixture of riboflavin and its leuco compound is −0 21 volts [42]

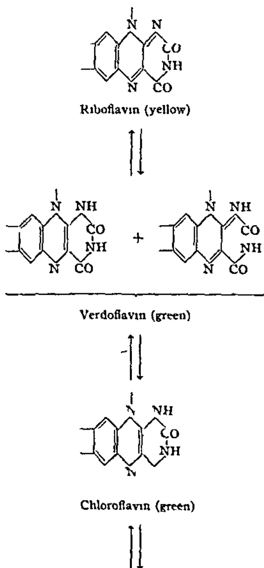
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[40] P Karrer H Salomon K Schöpp E Schlitter and H Fritzsche *Helv Chim Acta* 17 1010 (1934)

[41] R Kuhn P György and T Wagner Jauregg *Ber* 66 576 (1933)

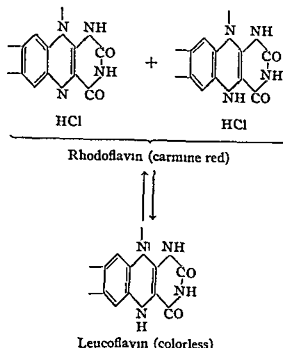
[42] E S G Barron and A B Hasting *J Biol Chem* 105 VII (1934) K Bierich and A Lang *Z physiol Chem* 223 180 (1934) K C Stern *Biochem J* 28 943 (1934) R Kuhn and G Moruzzi *Ber* 67 1220 (1934) R Kuhn and P Boulanger *Ibid* 69 1557 (1936) F J Stare *J Biol Chem* 112 223 (1935)

It has been postulated that by reduction of riboflavin to the leuco compound, three intermediate compounds result, which consist of molecular compounds of reduced and unreduced molecules [48] *Verdoflavin* consists of riboflavin and monohydro riboflavin, *chloroflavin* is a quinhydrone of riboflavin and leuco riboflavin, and *rhodoflavin* hydrochloride contains the hydrochlorides of leuco riboflavin and monohydro riboflavin




(Formula continued on following page)

[48] R Kuhn and R Ströbel *Ber* 70 753 (1937)

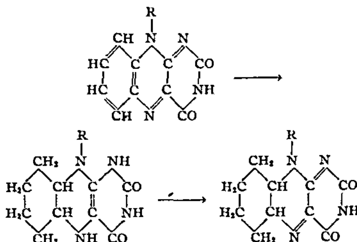


Rhodoflavin (carmine red)

Leucoflavin (colorless)

The existence of these intermediate stages is somewhat dubious, since titration curves of the reduction process indicate the presence of only one intermediate.[44] Since the quinhydrone is the only form that exists in *diluted* aqueous solution, it seems reasonable to assume that only this form occurs under physiological conditions.

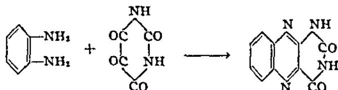
By energetic catalytic hydrogenation of flavins, octahydro flavins are obtained[45] which are easily oxidized in alkaline solution by air to the corresponding hexahydro flavins.



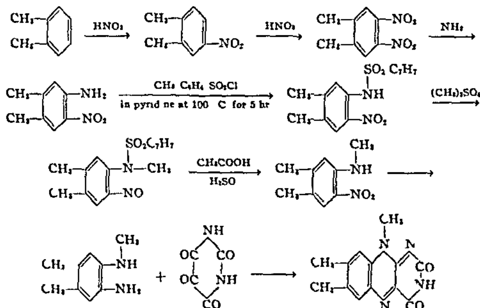
[44] L. Michaelis and G. Schwarzenbach, *J. Biol. Chem.*, 123, 527 (1938).
[45] P. Karrer and R. Ostwald, *Rec. trav. chim.*, 57, 500 (1938).

## 7 Synthesis of Vitamin $B_2$ and Other Flavins

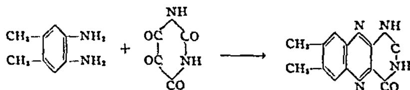
Alloxazines were first synthesized by Kuhling[46] by condensation of the hydrochloride of *o* phenylene diamine with alloxan



Kuhn and co-workers[47] synthesized lumiflavin from alloxan and 1,2 dimethyl-4 amino 5 methyl amino benzene in acid solution The *N* methyl *o* xylylene diamine was obtained according to the following scheme



Karrer and co workers[48] synthesized lumichrome similarly

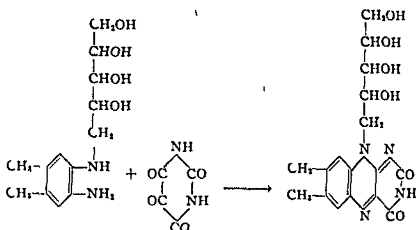


[46] O Kühling *Ber* 24 2363 (1891) 27 2116 (1894) 28 1968 (1895) O Kühling and O Kaselitz *Ibid* 39 1314 (1906)

[47] R Kuhn K Reinemund and F Weygand *Ibid* 67 1460 (1934) R Kuhn and K Reinemund *Ibid* 67 1932 (1934) R Kuhn H Rudy and K Reinemund *Ibid* 68, 170 (193 )

[48] P Karrer H Salomon K Schöpp F Schlitter and H Fritzsche *Helv Chim Acta* 17 1010 (1934)

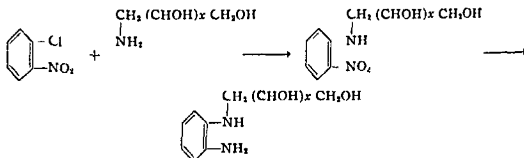
According to the same principle, riboflavin is obtained from alloxan and dimethyl amino phenyl ribamine




The condensation of the 4,5 dimethyl 2 amino phenyl ribamine with alloxan is carried out in acid solution When boric acid is used as catalyst in acetic acid solution the yield of the condensation product increases considerably [49]

The synthesis of the dimethyl amino phenyl ribamine has been achieved by the following methods

1 *o* Nitro chloro benzenes are condensed with amino sugars and the reaction product hydrogenated to the diamine [50] This method gives satisfactory yields when the sugar compound contains two or three hydroxyl groups, but poor yields with sugars containing four and five hydroxyl groups



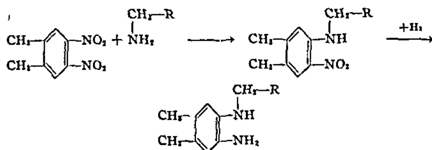

2 *o* Dinitro xylene is condensed with ribamine in aqueous alcoholic solution and catalytically reduced to the corresponding diamine [51] The

[49] R Kuhn and F Weygand *Ber* 68 1282 (193 ) See also R Kuhn and A H Cook *Angew Chem* 49 6 (1936)

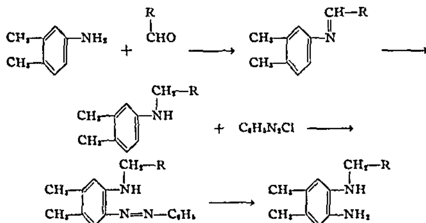
[50] P Karrer H Salomon K Schöpp and I Schlitter *Helv Chim Acta* 17 116 (1934) P Karrer I Schlitter K Pfaehler and F Benz *Ibid* 17 1516 (1934)

[51] R Kuhn and F Weygand *Ber* 68 1001 (193 )

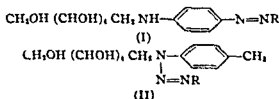
over all yield of riboflavin according to this process is 4 5% of the ribose used



3 3 4 Xylidene[52] is condensed with ribose and the formed riboside is catalytically reduced The second amino group is introduced by coupling with diazonium salts to form azo dyes which by reduction yield the desired diamine[53]



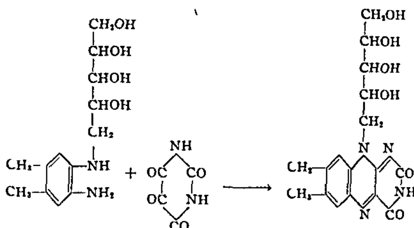
This method gives very high yields on riboflavin (38%) calculated from the required ribose but cannot be used for the preparation of any other flavin Only *m p* disubstituted aniline derivatives couple with diazonium salts in *o* position Phenyl glucamine for example couples in *p* position, yielding (I) *p* toluene glucamine yields (II)



[52] P Karrer B Becker F Benz P Frei H Salomon and K Schöpp *Helv Chim Acta* 18, 1435 (1935) W A Wisansky and S Ansbacher *J Am Chem Soc* 63 2532 (1941)

[53] P Karrer and H Meerwein *Helv Chim Acta* 18 1130 (1935) 19 264 (1936)

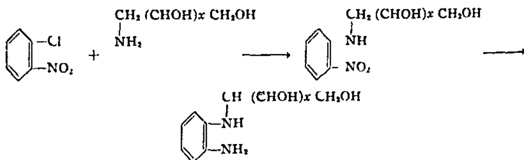
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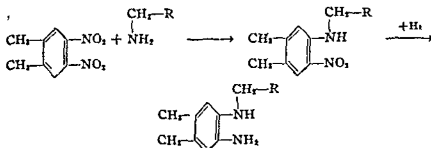

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R Kuhn and F Weygand *Ber* 68 1782 (1935) See also R Kuhn and A H Cook *Angew Chem* 49 6 (1936)

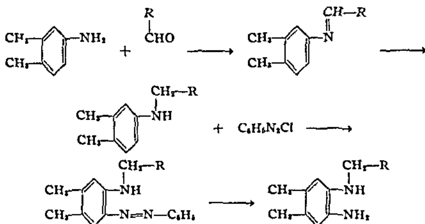
[50] P Karrer H Salomon K Schöpp and E Schlitter *Helv Chim Acta* 17 116 (1934) P Karrer E Schlitter K Pfaehler and F Benz *Ibid* 17 1 16 (1934)

[51] R Kuhn and F Weygand *Ber* 68 1001 (1935)

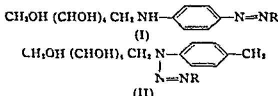
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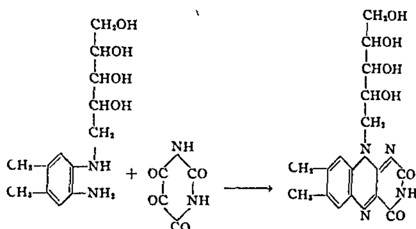

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[52] P Karrer B Becker F Benz P Frei H Salomon and K Schöpp *Helv Chim Acta* 18, 1435 (1935) W A Wisansky and S Ansbacher *J Am Chem Soc* 63 2532 (1941)

[53] P Karrer and H Meerwein *Helv Chim Acta* 18, 1130 (1935) 19 264 (1936)

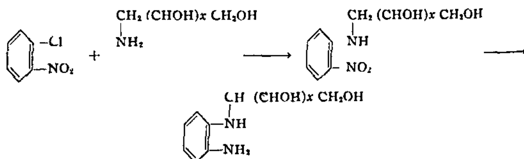
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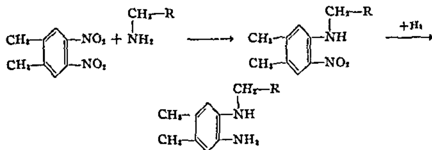

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[49] R Kuhn and F Weygand *Ber* 68 1282 (1935) See also R Kuhn and A H Cook *Angew Chem* 49 6 (1936)

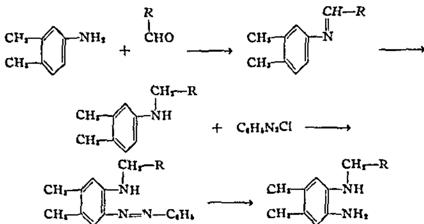
[50] P Karrer H Salomon K Schöpp and F Schlitter *Helv Chim Acta* 17 1165 (1934) P Karrer F Schlitter K Pfaehler and F Benz *Ibid* 17 1516 (1934)

[51] R Kuhn and F Weygand *Ber* 68 1001 (1935)

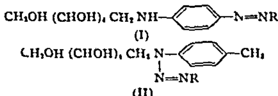
over all yield of riboflavin according to this process is 4 5% of the ribose used




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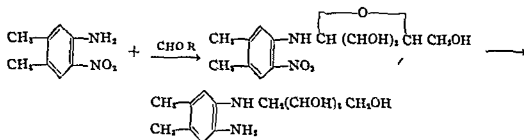

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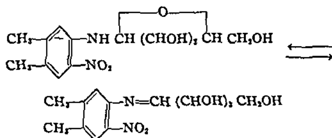

[52] P Karrer B Becker F Benz P Frei H Salomon and K Schöpp *Helv Chim Acta* 18 1435 (1935) W A Wisansky and S Ansbacher *J Am Chem Soc* 63 2532 (1941)

[53] P Karrer and H Meerwein *Helv Chim Acta* 18 1130 (1935) 19 264 (1936)

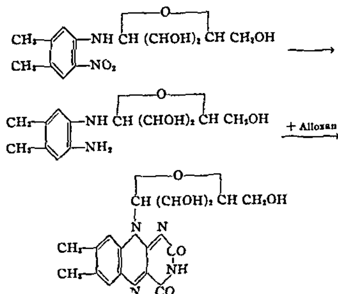
4 *o* Nitro xylidine is condensed with ribose and the reaction product is catalytically reduced to the diamine[54] This method yields 16% riboflavin calculated on the amount of ribose used



The intermediate is an amino glucoside which exists in an equilibrium with the tautomeric Schiff base



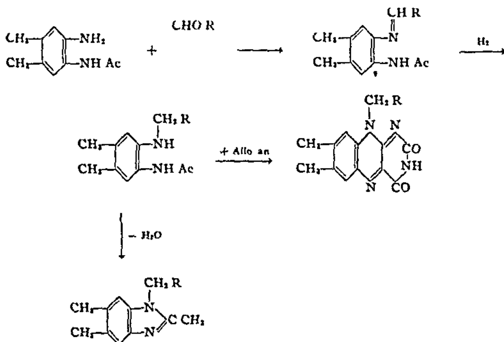
The Schiff base can also be reduced partially to the *o* phenylene diamine glucoside which after acetylation of the free hydroxyl groups may be con



[54] R Kuhn and R Ströbele *Ber* 70 773 (1937) R Kuhn K Reinemund F Weygand and R Ströbele *Ibid* 68 176 (1935)

densed with alloxan to the corresponding acetylated flavin glucoside[65] The latter upon saponification yields the free glucoside Flavin glucosides do not exhibit the physiological properties of vitamin $B_2$

5 *N* Mono acyl *o* phenylene diamines condense with sugars to the Schiff bases which are reduced simultaneously to the *N* (*o* acyl amino phenyl) amino sugars[66] These are condensed in acid solution with alloxan to the corresponding flavins The acyl groups are automatically saponified during the reaction The yields on flavins according to this process are extremely low, since the main reaction is an intermolecular dehydration which yields benzimidazole derivatives



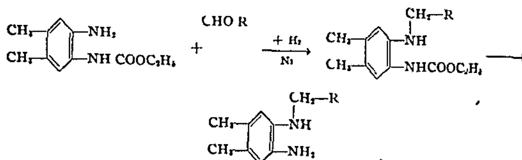

6 In the preceding method one amino group is temporarily protected by acetylation while the other amino group is condensed with ribose A better method is the protection of the amino group by the carbethoxy group[67]

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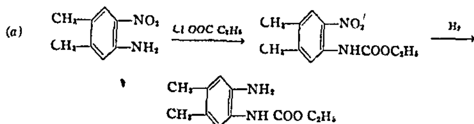
[65] R Kuhn and R Ströbele *Ber* 70 74 (1937)

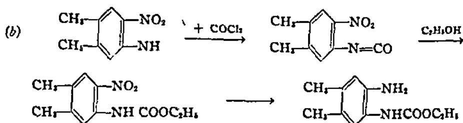
[66] P Karrer K Schöpp F Benz and K Pfaehler *Helv Chim Acta* 18 69 (1935) *Ber* 68 216 (1935)

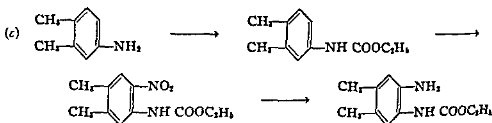
[67] P Karrer K Schöpp F Benz and K Pfaehler *Helv Chim Acta* 18 69 (193 ) P Karrer K Schöpp and F Benz *Ibid* 18 426 (1935) H v Euler P Karrer H Malmberg K Schöpp F Benz B Becker and P Frei *Ibid* 18 2 (193 ) P Karrer H Salomon K Schöpp F Benz and B Becker *Ibid* 18 908 1143 1435 (1935) P Karrer and F M Strong *Ibid* 18 1343 (1935)



The urethane can be prepared by one of the following procedures





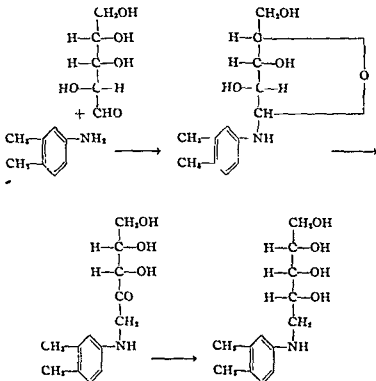


The yield of riboflavin according to this procedure is approximately 14–15% of the ribose used

7 While in the procedures described under (1 to 6) *d* ribose has been used as an intermediate, it is also possible to use some other sugar derivatives A very elegant method[56a] for the preparation of dimethyl amino phenyl ribamine is to condense 3,4 xylidine with *d* arabinose in the presence of small amounts of acid to form the *d* arabinoside which upon heat

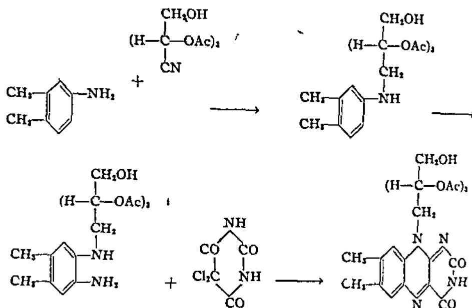
[56a] F Weygand *Ber* 73 1259 (1940)

ing to 75° C undergoes an Amadori rearrangement to the *d* iso arabinos amine Upon catalytic hydrogenation in alkaline solution the *d* iso arabinosamine is converted into 3 4 dimethyl phenyl *d* ribamine This compound is obtained in about 13% of theory from the arabinose originally used The arabamine is then coupled with a diazonium salt and reduced to the desired diamine as described under (3)




8 3,4 Xylidine is condensed[86b] reductively with tetra acetyl ribono nitrile which is obtained from ribonic acid via the amide The dimethyl phenyl ribamine is then coupled with para nitrophenyl diazonium chloride and reduced to N tetra acetyl ribitylamino 2 amino-4 5 dimethyl benzene according to the procedure described under (3) It has been recommended to condense the last mentioned compound with 5 5′ dichloro barbituric acid to obtain vitamin $B_2$ instead of using alloxan as has been described previously

[86b] M Tishler and J W Wellman U S P 2,261 608



## 8 Industrial Methods of Preparation

The relative technical importance of natural *versus* synthetic vitamin $B_2$ is dependent on the use for which the vitamin is intended For animal foods, various riboflavin preparations from natural sources such as yeast, whey or anaerobic growing fermentation bacteria, for example, from butyl alcohol fermentations are used The riboflavin content of these preparations varies considerably and must be standardized For human therapy, the use of pure riboflavin is preferred Since the isolation of pure riboflavin is more expensive than the synthesis thereof, synthetic vitamin $B_2$ is generally used clinically, especially in cases of an exclusive vitamin $B_2$ deficiency

The synthesis of vitamin $B_2$ is carried out according to the methods described in the preceding section The starting materials are *o* xylene *d* ribose and alloxan *o* Xylene is available as a by product from the petroleum industry *d* Ribose is obtained either from natural sources or by synthesis A convenient method for the preparation of small amounts of *d* ribose is the hydrolysis of yeast nucleic acid [58] Synthetic *d* ribose is prepared from *d* glucose [59, 60] Alloxan is usually obtained by oxidation of uric acid or barbituric acid

[58] H Bredereck *Ber* 71 408 (1938) H Bredereck M Köthnig and F Berger *Ibid* 73 956 (1940)
[59] W C Austin and F I Humoller *J Am Chem Soc* 56 1152 (1934)
[60] R Kuhn K Reinemund F Weygand and R Ströbele *Ber* 68 1765 (1935)

## 9 Flavin-Enzymes

### (a) *Enzyme Systems Containing Riboflavin*

Riboflavin takes part in a number of different enzyme systems in tissues These systems consist of an apoenzyme and a coenzyme The apoenzyme is a specific protein and is also called pheron bearer protein or Zwischenferment The coenzyme constitutes the prosthetic group of the enzyme system and contains riboflavin as part of its constitution There are two different riboflavin-containing coenzymes namely a mononucleotide and a dinucleotide The mononucleotide has the constitution of a riboflavin phosphate while the dinucleotide is a riboflavin adenine dinucleotide (For details see page 177 ) These coenzymes combine with different apoenzymes to carry out specific reactions The same coenzyme can serve as the prosthetic group of a number of different apoenzymes The function of all enzyme systems is to transfer hydrogen for example in the carbohydrate and amino acid metabolism In the carbohydrate metabolism for example the substrate is oxidized by dehydrogenation through the nicotinamide containing enzymes which are reduced in this process to the dihydro compounds which in turn are oxidized by the riboflavin containing enzymes Thus the dihydro nicotinamide enzymes serve as substrates for the riboflavin enzymes which are converted into dihydro derivatives (see page 180) The dihydro compounds are then reoxidized to the corresponding riboflavin coenzymes by a number of specific

TABLE I

| Enzyme | Source | Prosthetic group | Hydrogen donor | Hydrogen acceptor |
|---|---|---|---|---|
| Old yellow enzyme | Bottom yeast | Mononucleotide | Dihydro coenzymes I and II | Molecular oxygen |
| Cytochrome *c* reductase | Top yeast | Mononucleotide | Dihydro coenzyme II | Cytochrome *c* |
| Diaphorase I | Heart | Dinucleotide | Dihydro-coenzyme I | Cytochrome (*a* and) *b* |
| Diaphorase II | Bottom yeast adrenal gland | Dinucleotide | Dihydro-coenzyme II | ? |
| Diaphorase } | Milk | Dinucleotide | Dihydro-coenzyme I (and II?) | ? |
| Aldehyde oxidase } | Milk | Dinucleotide | Aldehyde | ? |
| Xanthine oxidase } | Milk | Dinucleotide | Xanthine | |
| Aldehyde oxidase | Liver | Dinucleotide | Aldehydes | ? |
| *d*-Amino-acid oxidase | Kidney liver[4] | Dinucleotide | *d* Amino-acids | ? |
| Glucose oxidase | Yeast | ? | Glucose | ? |
| ? | Bottom yeast | Dinucleotide | ? | Fumaric acid |
| Diamine oxidase | Kidney | ? | Diamines and polyamines | ? |
| ? | Top yeast | Dinucleotide[41] | ? | ? |

[4] D E Green W F Knox and P K Stumpf *J Biol Chem* 138 775 (1941)

reactions Thus, the hydrogen may be transferred to fumaric acid or may react directly with oxygen or indirectly through the cytochromes *a*, *b* or *c* The different reactions which are catalyzed by the riboflavin containing coenzymes and which have been elucidated are summarized in Table I

Some of the enzymes listed in Table I apparently have a second pros thetic group in addition to the riboflavin coenzyme This other compound is characterized by a brownish color but has not been identified It has been observed in the flavin enzymes isolated from milk (diaphorase, alde hyde and xanthine oxidase), from liver (aldehyde oxidase) and from top yeast (action not characterized) Whether or not the group with the brownish color is the same in these three flavin enzymes has not been estab lished

The reactions catalyzed by the flavin enzymes as indicated in Table I are discussed more fully below

**Oxidation of the Codehydrogenases I and II** This reaction can be accomplished by enzymes containing either the riboflavin mono or di nucleotide in the presence of specific apoenzymes The mononucleotide containing flavin enzyme is the 'old' yellow enzyme of Warburg and Chris tian [62] It is purified by adsorption methods[63, 64] or by cataphoresis,[65] has a molecular weight of about 70,000[66] and a redoxy potential of −0 06 volts at pH 7 0 and 20° C It is split into the apoenzyme and the coenzyme by denaturation of the apoenzyme with methanol or by dialysis against dilute hydrochloric acid A resynthesis of the enzyme from the apo enzyme and the coenzyme can be accomplished Riboflavin, if added to the apoenzyme in large excess, forms an active flavin enzyme[67] although the union is not quite as firm as with the mononucleotide The riboflavin adenine dinucleotide is also able to combine with the specific apoenzyme and to react like the 'old yellow enzyme The chemistry of the apo enzyme is largely unknown but by hydrolysis the following amino acids have been obtained in a total yield of 65% arginine histidine lysine, proline tyrosine, phenyl alanine, tryptophane, cystine and glutamic acid [68] The 'old' yellow enzyme dehydrogenates codehydrogenases I and II and

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[62] O Warburg and W Christian *Naturwissenschaften* 20 688 980 (1932) *Biochem Z* 254 438 (1932) 257 492 (1933) 266 377 (1933)

[63] F Weygand and H Stocker *Z physiol Chem* 247 167 (1937)

[64] F Weygand and L Birkofer *Ibid* 261 172 (1939)

[65] H Theorell *Biochem Z* 275 37 344 (1934) 278 263 (1935)

[66] H Theorell *Ibid* 278 279 (1935) R A Kekwick and K O Pederson *Biochem J* 30 2201 (1936)

[67] R Kuhn and H Rudy *Ber* 69 2557 (1936)

[68] R Kuhn and P Desnuelle *Ibid* 70 1907 (1937)

the dihydro form is dehydrogenated by oxygen The rate of the last reaction is a function of the partial pressure of the oxygen present In animal tissues the oxygen tension is so low that the oxidation can hardly be accomplished Thus this reaction of the reduced riboflavin coenzyme with oxygen demonstrates a chemical function of the molecule but not its real physiological action The reaction product of oxygen with the reduced riboflavin coenzyme is hydrogen peroxide which destroys the life of the cells The latter has been demonstrated in the case of anaerobic growing lactic acid bacteria Thus, if the old yellow enzyme plays an essential function in the living organism an oxygen transporting system must take care of the dehydrogenation of the dihydro riboflavin enzyme It has been observed that cytochrome *c* reacts with the reduced 'old yellow enzyme, but this reaction is too slow to be considered of physiological importance [69, 70] Fumaric acid[71, 72] and glyoxal[73] have been suggested as substances capable of oxidizing the dihydro riboflavin system but convincing experimental evidences to substantiate these conceptions have not been reported

An enzyme containing the riboflavin mononucleotide but an apoenzyme different from that of the old yellow enzyme has been found in the cytochrome *c* oxidase This enzyme catalyzes the dehydrogenation of the code hydrogenase II and the reduction of the oxidized form of cytochrome *c*[74] under physiological conditions

Besides the enzyme systems which contain riboflavin mononucleotide and react with the dehydrogenases there are enzyme systems which contain the dinucleotide and which are capable of carrying out the same reactions They are called the diaphorases [75] coenzyme factor [76] or pyridine nucleotide oxidase and occur in animal and in plant tissues [77] The enzymes from heart skeletal muscle and yeast are apparently identical [78, 79, 80] The same enzyme or enzymes also occur in bacteria [81] There are two dif

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[69] H Theorell *Angew Chem* 51 738 (1938)

[70] H Theorell *Nature* 138 687 (1936) *Biochem Z* 288 31 (1936) 279 463 (1935)

[71] *E Adler and H v Euler A kiv Kemi Mineral Geol B12 No 36 (1937)*

[72] A Szent Györgyi *Z physiol Chem* 244 105 (1936)

[73] T Bersin *S B Ges Bef ges Naturw Marburg* 71 56 (1936)

[74] F Haas B L Horecker and T R Hogness *J Biol Chem* 136 747 (1940)

[75] H v Euler and H Hellström *Z physiol Chem* 252 31 (1938) H v Euler and K Hasse *Naturwissenschaften* 26 187 (1938) H v Euler and G Günther *Ibid* 26 676 (1938)

[76] J G Dewan and D F Green *Biochem J* 32 626 (1938) F B Straub H S Corran and D E Green *Nature* 143 76 119 (1939)

[77] E B Lockhart *Biochem J* 33 613 (1939)

[78] F B Straub *Ibid* 33 787 (1939)

[79] H S Corran D E Green and F B Straub *Ibid* 33 793 (1939)

[80] F Haas *Biochem Z* 298, 378 (1938)

[81] D E Green and J G Dewan *Biochem J* 32 626 (1938)

ferent diaphorases, namely, diaphorase I which dehydrogenates codehydrogenase I, and diaphorase II, which dehydrogenates codehydrogenase II[82] The reversed reaction, the dehydrogenation of the reduced diaphorases, is probably carried out by cytochromes *a* and *b* It has been shown experimentally that diaphorase I reacts with cyctochrome *b*[83] and some evidence has been obtained for the participation of cyctochromes *a* and *b* in the dehydrogenation of the diaphorases[84]

The apoenzyme of the diaphorase I has a molecular weight of about 70,000 It appears that the combination of the apoenzyme with the coenzyme is rather loose and that the coenzyme may change from one apoenzyme molecule to another with considerable speed On the other hand, the same specific protein may possibly serve as apoenzyme for both the coenzymes of the riboflavin and the nicotinamide type[85] One mol of the diaphorase catalyzes the oxidation of about 8000 mols of codehydrogenase I under optimum conditions in a synthetic system containing methylene blue as an oxygen carrier[86]

Through the codehydrogenases the riboflavin enzyme systems take part[87] in the reactions carried out by the nicotinamide containing enzymes[88] (see page 227), such as in the dehydrogenation of hexose monophosphate in yeast[89] and muscle,[90] of alcohol in yeast,[91] of glucose in liver,[92] of malic acid in muscle,[93] of lactic acid in muscle,[94] of citric acid in muscle and in plant cells,[95] of dioxy acetone phosphate in yeast,[96] of glycerin aldehyde phosphate in yeast[97] and of glycerin phosphoric acid in seeds[98]

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[82] E Adler H v Euler G Günther and E D Plass *Skand Arch Physiol* 82 61 (1939) E Adler H v Euler and G Günther *Nature* 143 641 (1939) E P Abraham and E Adler *Biochem J* 34 119 (1940)

[83] K Okunuki and E Yakusizi *Proc Imp Acad* (*Tokyo*) 16 144 (1940)

[84] J G Dewan and D E Green *Biochem J* 32 626 (1938)

[85] E Haas *Biochem Z* 290 291 (1937)

[86] E Haas *Ibid* 298 378 (1938) H S Corran D E Green and F B Straub *Biochem J* 33 793 (1939)

[87] H Theorell *Ergeb Enzymforsch* 6 132 (1937) O Warburg *Ibid* 7 210 (1938)

[88] O Warburg W Christian and H Griese *Biochem Z* 282 157 (1935)

[89] O Warburg and W Christian *Ibid* 254 438 (1932) 257 492 (1933) 266 377 (1933)

[90] T Wagner Jauregg *Z physiol Chem* 231 55 (1935)

[91] H v Euler and E Adler *Ibid* 226 195 (1934)

[92] E Adler and H v Euler *Ibid* 232 6 (1935)

[93] T Wagner Jauregg *Ibid* 228 273 (1934) 231 55 (1935) E Adler and M Michaelis *Ibid* 238 261 (1935)

[94] E Adler and M Michaelis *Ibid* 235 154 (1935) 238 261 (1936) T Wagner Jauregg and E F Möller *Ibid* 236 216 (1935)

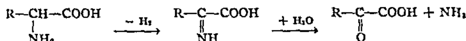
[95] T Wagner Jauregg *Ibid* 233 215 (1935)

[96] H v Euler E Adler and H Hellström *Ibid* 241 239 (1936)

[97] H v Euler E Adler and H Hellström *Ibid* 241 239 (1936)

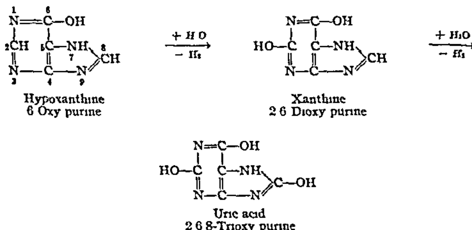
[98] T Wagner Jauregg and H Rauen *Ibid* 237 233 (1935)

**Oxidation of *d*-Amino-acids** The oxidation of the antipodes of the naturally occurring amino acids into oxo acids is carried out by an enzyme system containing riboflavin adenine dinucleotide[99–101] according to the following reaction scheme [102]



One mol of the riboflavin adenine dinucleotide transports in the presence of an excess of the apoenzyme approximately 2000 mols of oxygen per minute at 38° C The apoenzyme has a molecular weight of about 65 000 The coenzyme is highly dissociated from the apoenzyme in solution but the reduced coenzyme apparently is not dissociated

**Oxidation of Xanthine** By means of an enzyme system containing riboflavin adenine dinucleotide plus an unknown colored compound several purines, primarily xanthine and hypoxanthine, are dehydrogenated to uric acid [103–106]



Hypoxanthine
6 Oxy purine

Xanthine
2 6 Dioxy purine

Uric acid
2 6 8-Trioxy purine

The conversion of hypoxanthine to xanthine and the conversion of xanthine to uric acid require the presence of the same riboflavin enzyme One mol

[99] O Warburg and W Christian *Biochem Z* 295 261 (1938) 298 150 (1938)
[100] E Negelein and H Brömel *Ibid* 300 225 (1939)
[101] F B Straub *Nature* 141 603 (1938)
[102] H A Krebs *Z physiol Chem* 217 191 (1933) 218 157 (1933) *Biochem J* 29 1620 (1935)
[103] E G Ball *Science* 88 131 (1938) *Angew Chem* 51 738 (1938) *J Biol Chem* 128 51 (1939)
[104] H S Corran and D E Green *Biochem J* 32 2231 (1938)
[105] D E Green and H S Corran *Angew Chem* 51 738 (1938)
[106] H S Corran J G Dewan A H Gordon and D E Green *Biochem J* 34, 1694 (1939)

of the coenzyme catalyzes the oxidation of about 300 mols of hypoxanthine or xanthine per minute The oxidation rate of other purines is considerably slower [107]

The same enzyme system is also able to effect an anaerobic dismutation of xanthine to hypoxanthine and uric acid



2 xanthine ⇌ hypoxanthine + uric acid

This is an equilibrium reaction and xanthine can therefore be formed from uric acid and hypoxanthine [108, 109]

This riboflavin containing enzyme xanthine oxidase, occurs for example, in milk and liver The apoenzyme has a molecular weight of about 280,000[110] and probably binds two riboflavin groups per molecule in addition to some other unknown colored group which may act as coenzyme The apoenzyme is different from those of the other riboflavin containing enzyme systems

**Oxidation of Aldehydes** The enzyme which catalyzes the oxidation of aldehydes to carboxylic acids, for example, propionic aldehyde to propionic acid, was originally observed in milk and is called aldehyde oxidase or Schardinger enzyme [111] The aldehyde oxidase from liver[112, 113] contains the riboflavin adenine dinucleotide and is specific for the oxidation of aldehydes The aldehyde oxidase from milk also acts as xanthine oxidase and as dihydro codehydrogenase I oxidase Whether or not these enzymes are identical has not been decided There is also a discrepancy of opinion as to whether or not the aldehyde oxidase and xanthine oxidase from liver are a single compound or a mixture of compounds Much evidence has accumulated which favors the view that a single compound acts as an enzyme in milk for the oxidation of both xanthine and aldehydes [114, 115] If that is the case, the aldehyde oxidase from liver must be a different compound

**Oxidation of Diamines** A riboflavin containing coenzyme of unknown composition takes part in the enzymatic conversion of di and poly

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107 M Dixon *Enzymologia* 5 198 (1938) L Reichel and W Burkart *Z physiol Chem* 260 135 (1939)

108 D E Green *Biochem J* 28 1550 (1934)

109 S Filitti *J chim phys* 32 1 (1935)

110 J S L Philpot *Biochem J* 32 2240 (1938)

111 F Schardinger *Z Untersuch Nahr u Genussm* 5 1113 (1902)

112 V Subrahmanyan D E Green and A H Gordon *Nature* 144 1016 (1939)

113 A H Gordon D E Green and V Subrahmanyan *Biochem J* 34 764 (1940)

114 V H Booth *Ibid* 32 494 (1938)

115 M Dixon *Enzymologia* 5 198 (1938) L Reichel and W Burkart *Z physiol Chem* 260 135 (1939)

amines into amino aldehydes [116] This action is similar to the oxidation of d amino acids, but the enzymes (at least the apoenzymes) involved appear to be different The amines studied in this reaction are primarily histamine, cadaverin and spermin

**Oxidation of Glucose** The oxidation of glucose to gluconic acid in yeast is carried out with the aid of an enzyme which contains riboflavin [117, 118] The manner in which riboflavin is bound in this enzyme system is not known

**Reduction of Fumaric Acid** In yeast fumaric acid is reduced to succinic acid by an enzyme system which contains riboflavin adenine dinucleotide [119] Per mol of dinucleotide 2000–3000 mols of hydrogen are transported per minute This enzyme is also able to reduce other compounds such as maleic acid crotyl alcohol phenyl crotyl alcohol and geraniol but the rate of reduction is less than $^1/_{60}$ that of the rate of fumarate reduction The specificity of the apoenzyme has not been determined but it appears probable that the apoenzyme of at least one of the oxidases previously discussed is identical with that of the fumaric hydrogenase

**Reduction of Cytochrome *a*, *b* and *c*** The reduction of the oxidized form of the cytochromes is linked with the oxidation of the dihydro codehydrogenases (see page 174)

### (*b*) *Coenzymes Containing Riboflavin*

**Riboflavin-5′-phosphoric Acid (Riboflavin-mononucleotide)** Riboflavin 5′ phosphoric acid was first obtained from heart muscle and was characterized as a yellow water soluble dye This compound was given the name "cytoflav" [120] The position of the phosphoric acid in the riboflavin molecule was established by oxidation with periodic acid [121] Formaldehyde was not obtained as a reaction product as would be expected in case a free primary hydroxyl group should be present in the 5′ position This result was confirmed by synthesis which was carried out according to the following scheme [122]

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[116] E A Zeller R Stern and M Wenk *Helv Chim Acta* 23 1 (1940)
W Franke and M Deffner *Ann* 541 117 (1939)

[117] D Mueller *Biochem Z* 199 136 (1928) 205 111 (1929) 213 211 (1929) 232 423 (1931)
W Franke and M Deffner *Ann* 532 1 (1937)

[118] F G Fischer A Roedig and K Rauch *Naturwissenschaften* 27 197 (1939) F G Fischer and H Eysenbach *Ann* 529 87 (1937) 530 99 (1937)

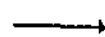
[119] I Banga and A Szent Györgyi *Biochem Z* 246 203 (193 )

[120] P Karrer P Frei and H Meerwein *Helv Chim Acta* 20 79 (1937)

[121] R Kuhn H Rudy and F Weygand *Ber* 69 1543 (1936) R Kuhn and H Rudy *Ibid* 69 1974 (1936)

$CH_2\,CHOH\,CHOH\,CHOH\,CH_2OH$

Riboflavin



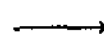
$CH_2\,CHOH\,CHOH\,CHOH\,CH_2OC(C_6H_5)_3$

5′ Trityl riboflavin



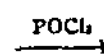
$CH_2$ CHOAc CHOAc CHOAc $CH_2$O Trityl

2′ 3′ 4′ Triacetyl 5′ trityl riboflavin



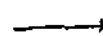
$CH_2$ CHOAc CHOAc CHOAc $CH_2OH$

2′ 3′ 4′ Triacetyl riboflavin




$CH_2$ CHOAc CHOAc CHOAc $CH_2OPO_3H_2$

2′ 3′ 4′ Triacetyl riboflavin 5′ phosphate



$CH_2\,CHOH\,CHOH\,CHOH\,CH_2OPO_3H_2$

Riboflavin 5′ phosphate



The phosphorylation of riboflavin *in vitro* has also been carried out by a phosphatase concentrate prepared as a dry powder from the intestinal mucosa of rats, cats and pigs,[123] and by a glycerol extract of the small intestine of rats in 0 01 molar phosphate solution [124]

The properties of riboflavin 5′ phosphoric acid correspond closely to those of the free riboflavin The ester is considerably more soluble in water than the free riboflavin, and can be precipitated in the form of various salts Lumiflavin and lumichrome, respectively, are formed upon irradiation similar to the formation of these compounds from riboflavin In cataphoresis experiments riboflavin does not move the phosphoric acid ester, however, migrates to the anode

In neutral solution, the ester is quite stable Hydrolysis occurs rapidly in acid solution, but considerably more slowly in alkaline medium The ester is also hydrolyzed by phosphatases such as the α glycero phosphatase

Riboflavin 5′ phosphoric acid combines with specific proteins the apoenzymes, by attachment at two points, namely, at the phosphoric acid group and at the slightly acidic imino group in 3 position of the riboflavin molecule In accordance with this conception, flavins which are substituted in the 3 position do not form enzyme systems[125] and are also devoid of vitamin activity Furthermore, the typical fluorescence of riboflavin is dependent upon the presence of a free 3 imino group and neither 3 substituted riboflavins nor the enzyme systems exhibit fluorescence

**Riboflavin-adenine-dinucleotide** The riboflavin adenine dinucleotide has the composition $C_{27}H_{33}O_{15}N_9P_2$ and forms salts such as a mono barium and mono sodium salt The constitution of the dinucleotide has not been well defined By enzymatic[126] or by acid[127] hydrolysis the dinucleotide is split into two mononucleotides namely, into riboflavin 5′-phosphoric acid and adenylic acid (adenosine 5′ monophosphoric acid) (See formula on following page )

The dinucleotide is widely distributed in animal tissues and in micro organisms It is also believed to occur in plants It has been isolated from liver kidney heart, muscles Jensen sarcoma (a special type of rat tumor) and yeast [128, 129] The isolation involves a denaturation of the apoen

[123] H Hübner and F Verzár *Helv Chim Acta* 21 1006 (1938) R Pulver and F Verzár *Enzymologia* 6 333 (1939)

[124] H Rudy *Naturwissenschaften* 23 286 (193 )

[125] R Kuhn and H Rudy *Ber* 69 ?5 7 (1936)

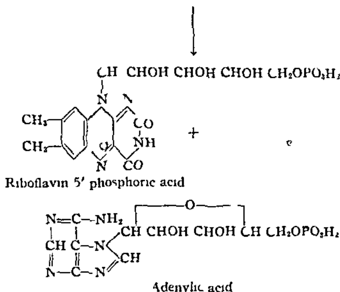
[126] O Warburg and W Christian *Biochem Z* 298 150 368 (1938)

[127] E P Abraham *Biochem J* 33 543 (1939)

[128] P Karrer P Frei and M Meerwein *Helv Chim Acta* 20 79 (1937) P Karrer I Frei B H Ringier and H Bendas *Ibid* 21 8 6 (1938)

[129] O Warburg W Christian and A Griese *Biochem Z* 295 ?1 (1938) 297 417 (1938) 298 150 (1938)

zyme by heat treatment (75° C) followed by extraction with phenol. From the phenol solution the dinucleotide is extracted with water in the presence of ether. From the acidified water solution the dinucleotide is precipitated as the silver salt and after conversion into the barium salt the latter is recrystallized from water.

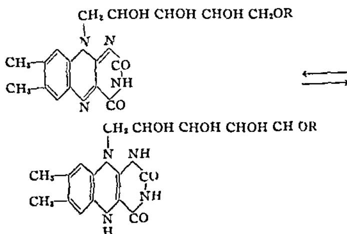


Riboflavin 5' phosphoric acid

Adenylic acid

### (c) *Mechanism of the Coenzyme Action*

Riboflavin acts in various enzyme systems by reversibly accepting and donating two atoms of hydrogen. This is accomplished by the addition of hydrogen to the 1 and 10 positions of riboflavin thus forming the previously discussed dihydro or leuco riboflavin (see page 160).



## 10 Specificity

Riboflavin is the only naturally occurring flavin with vitamin $B_2$ activity. A great number of flavin compounds have been prepared synthetically and have been tested for vitamin activity. Besides riboflavin the following compounds were found to exhibit vitamin $B_2$ activity:

7 Methyl 9 (*d*,1' ribityl) isoalloxazine [130]
6 Methyl 9 (*d* 1' ribityl) isoalloxazine [130]
6 Ethyl 7 methyl 9 (*d* 1' ribityl) isoalloxazine [131]

Whereas rats need about 5 γ of riboflavin per day they require about twice as much of these three compounds. Similar results are obtained when lactic acid bacteria are used for the evaluation of the vitamin activity [132]

The following compounds possess some stimulating activity for rats and lactic acid bacteria *in the presence of suboptimal amounts of riboflavin*

6 7 Dimethyl 9 (*l* 1' arabityl) isoalloxazine [133]
6,7 Dimethyl 9 (*d*,1' arabityl) isoalloxazine [134, 135]
7 Ethyl 9 (*d* 1' ribityl) isoalloxazine [136]
6 7 Trimethylene 9 (*l* 1' arabityl) isoalloxazine [137]
6 7 Tetramethylene 9 (*l* 1' arabityl) isoalloxazine [137]

Riboflavin tetra acetate and diacetone riboflavin are active on rats, probably due to hydrolysis in the organism. The tetra acetate is, however inactive on lactic acid bacteria. The 5 phosphoric acid ester of riboflavin[138] and the flavin adenine dinucleotide show full vitamin activity. The flavin glucosides are inactive.

Generally speaking substitution in 6 or 7 position is necessary for the vitamin activity. Absence of substituents in both positions is accompanied by high toxicity [139]. An unsubstituted imino group in 3 position is also necessary. Riboflavins which are alkylated in 3 position are totally devoid of vitamin and coenzyme activity.

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[130] P Karrer H v Euler M Malmberg and K Schöpp Svensk Kem Tid 47 153 (1935) P Karrer H Salomon K Schöpp F Benz and B Becker Helv Chim Acta 18 908 (193 )

[131] P Karrer and T H Quibell Helv Chim Acta 19 1034 (1936)

[132] P Karrer H Salomon K Schöpp E Schlittler and H Fritzsche Ibid 17 1010 (1934)

[133] H v Euler P Karrer M Malmberg K Schöpp F Benz B Becker and P Frei Ibid 18 52 (1935) P Karrer H v Euler M Malmberg K Schöpp and F Benz Svensk Kem Tid 47 9 (1935) P György Z Vitaminforsch 4 3 (1935)

[134] H v Euler P Karrer and M Malmberg Helv Chim Acta 18 1336 (1935)

[135] Vitamin activity questionable

[136] P Karrer and T H Quibell Helv Chim Acta 19 1034 (1936)

[137] R Kuhn H Vetter and H W Rzeppa Ber 70 1307 (1937)

[138] P György Proc Soc Exptl Biol Med 35 907 (1936)

[139] R Kuhn and P Boulanger Z physiol Chem 241 233 (1936)

## 11 Determination

### (a) Physical Methods

**1 Determination of the Absorption Spectrum** The determination of vitamin $B_2$ by its characteristic ultraviolet absorption spectrum is suitable only for solutions of the pure compound, and this method has the additional disadvantage that the vitamin is sensitive to light Thus during the determination the vitamin is at least partly destroyed, and the material destroyed before the beginning of the determination adds to the apparent value of the vitamin

**2 Determination of the Fluorescence Spectrum** Vitamin $B_2$ can be determined by its characteristic fluorescence spectrum[140] which shows a maximum at 565 mμ at pH 6 It is best to compare the intensity of the fluorescence with some standard such as pure riboflavin,[141] potassium dichromate,[142] fluorescein[143] or uranium glass [144] The fluorescence is directly proportional to the vitamin content Relatively reliable results have been obtained with this method when the vitamin $B_2$ content of animal tissues or milk is assayed The method becomes inaccurate however, in cases when other fluorescent substances are present as in the case of urine and yeast extracts Another source of error often encountered is a non quantitative extraction of the vitamin Such extractions are often necessary to separate riboflavin from interfering substances A method which has given results in agreement with biological assays consists in enzymatic hydrolysis of, for example, tissues, followed by extraction with water and determination of the fluorescence of the extract [145]

Several modifications of this basic assay procedure have been recommended Thus, vitamin $B_2$ has been determined indirectly by the fluorescence of the vitamin before and after reduction with sodium hydrosulfite [146] This has been possible since the reduced vitamin does not show the typical fluorescence of the unreduced vitamin Another modification is to determine, first the total fluorescence of a given solution and, second, the fluorescence of the interfering substances by destroying the vitamin through alkali [147]

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[140] H v Euler and E Adler *Svensk Kem Tid* 45 276 (1933) *Z physiol Chem* 223 105 (1934) G C Supplee S Ansbacher G E Flanigan and Z M Hanford *J Dairy Sci* 19 215 (1936)

[141] A Z Hodson and L C Norris *J Biol Chem* 131 621 (1939)

[142] A J Charite and N W Khaustov *Biochem J* 29 34 (1935) G N Murthy *Indian J Med Research* 24 1083 (1937)

[143] F H Cohen *Rec trav chim* 54 133 (1935) S M Weisberg and I Levin *Ind Eng Chem Anal Ed* 9 523 (1937)

[144] D B Hand *Ind Eng Chem Anal Ed* 11 306 (1939)

[145] F O Van Duyne *J Biol Chem* 139 207 (1941)

[146] A Z Hodson and L C Norris *Ibid* 131 621 (1939)

[147] H Kahler and E P Davis *Proc Soc Exptl Biol Med* 44 604 (1940)

3 Polarographic Determination It has been proposed to determine vitamin $B_2$ by the polarographic technique[148] Assays have been carried out so far only on relatively pure vitamin $B_2$ preparations and the method needs further study to determine whether or not this procedure can be applied for the determination of riboflavin in natural products

4 Lumi-Lactoflavin Method This method is based on the conversion of riboflavin into lumi lactoflavin by irradiation in alkaline solution. The so formed lumi lactoflavin is extracted from the acidified solution with chloroform and determined by its absorption in the fluorescence spectrometer[149] The accuracy of this method is limited, since the conversion of riboflavin into lumi lactoflavin is by no means quantitative Nevertheless this method proved to be effective if the conversion rate of pure riboflavin in a solution of about the same concentration as in the solution to be examined is determined separately taking care that the same pH, temperature and amount of air are present In another experiment a known amount of pure riboflavin is added to the unknown solution and the conversion is determined By proper correlation of the three figures obtained an accurate determination of the vitamin $B_2$ content of almost any solution can be achieved

*The separation of riboflavin from its phosphoric acid ester* is carried out by extraction of the aqueous solution with benzyl alcohol, in which only the free riboflavin is soluble[150]

*The separation of riboflavin and riboflavin 5′ phosphoric acid from flavin combined with proteins* is achieved by dialysis in cellophane tubes at 3° C for 16 hours

## (b) *Biological Methods*

1 Rat Growth Test The most accurate and generally accepted biological method for the determination of riboflavin is the curative growth test on rats[151 152 153] Young rats kept on a riboflavin free diet stop

[148] J J Lingane and O L Da s *J Biol Chem* 137 567 (1941)

[149] R Kuhn F Wagn r Jauregg and H Kaltschmitt *Ber* 67 1452 (1934) H v Euler B Adl r and A Schlötzer *Z phys l Chem* 226 87 (1934) F Vivanco *Naturwissenschafte* 23 306 (1935)

[150] A Emmerie *Natu e* 141 416 (1938) *Rec t av ch m* 58 290 (1939)

[151] A Bourqu n and H C Sherman *J Am Chem Soc* 53 3501 (1931)

[152] P György I W van Klaveren R Kuhn and T Wagner Jauregg *Z physiol Chem* 223 236 (1934)

[153] A. Bourqu n and H C Sherman *J Am Chem Soc* 53 3501 (1931) H E Mun ell *J Nut ition* 4 203 (1931) P György *Biochem J* 29 741 (1935) P L D y and W O Lang ton *J Nutrition* 7 97 (1934) G C. Supplee R C. Bender and O G Jen en *Ind Eng Chem Anal Ed* 11 495 (1939) T s. Hamilton and H H Mitch ll *J Nut ition* 10 117 (1935) L A Randoin A Raffy and J Aqu rrez lla *Compt end soc b ol* 126 872 (1937) H Lindholm *Biochem J* 32 314 (1938) E V Carl son nd H C Sherman *J Nut tion* 15 57 (1938) M M El Sadr T F Macrae and C. E Work *Biochem J* 34 601 (1940) J R Wagn r A F Axelrod M A Lipton and C A Elvehjem, *J Biol Chem* 136 357 (1940)

growing within a few days Addition of riboflavin to the diet causes continuation of growth The same principle has also been used in a prophylactic method

2 **Chick Growth Test** An apparently very reliable method is the growth response of chicks, which is within certain limits a linear function of the amount of riboflavin administered[154]

3 **Lactic Acid Bacteria Test** This method[155] is based on the essential nature of riboflavin for the growth of *Lactobacillus casei* The evaluation is carried out by measurement of the turbidity produced by the growth of the organism or by titration of the lactic acid formed This method determines not only riboflavin in the free form, but also in the combined forms (coenzymes and enzymes)[156,157]

### (c) *Biochemical Methods*

1 **Yellow Enzyme Test**[158] The oxygen uptake of the following system is measured Hexose-phosphoric acid, codehydrogenase II (page 235), apodehydrogenase and the specific protein of the yellow enyzme The rate of the oxygen uptake per minute is a function of the amount of the riboflavin added to this system

2 **Determination of Riboflavin-adenine-dinucleotide**[159] The dinucleotide is determined by its ability to act in combination with a specific protein as *d* amino acid oxidase[160,161,162] Actually, the rate of oxygen uptake is measured during the oxidation and is compared with the rate of oxygen uptake catalyzed by known amounts of the dinucleotide in the same system

## 12 Standards

One B S Rat Unit (Bourquin Sherman Unit) is defined as the daily amount of riboflavin required by rats to insure proper growth (10 g increase of body weight per week for two to four weeks)

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[154] T H Jukes *J Nutrition* 14 223 (1937)

[155] E E Snell and F M Strong *Ind Eng Chem Anal Ed* 11 346 (1939) *Enzymologia* 6 186 (1939) E E Snell F M Strong and W H Peterson *Biochem J* 31 1789 (1937) *J Am Chem Soc* 60 2875 (1938)

[156] R E Feeney and F M Strong *Proc Am Soc Biol Chem* 1940 XXXI

[157] F M Strong R E Feeney B Moore and H T Parsons *J Biol Chem* 137 363 (1941)

[158] R Kuhn and H Rudy *Ber* 69 25 7 (1936)

[159] O Warburg and W Christian *Biochem Z* 298 150 368 (1938)

[160] E Negelein and H Brömel *Ibid* 300 225 (1939)

[161] J R Klein and H I Kohn *J Biol Chem* 136 177 (1940)

[162] S Ochoa and R J Rossiter *Biochem J* 33 2008 (1939)

An International Unit of riboflavin has not been established Von Euler[163] proposed as International Unit 5 $\gamma$ of pure crystallized riboflavin, which amount produces an increase in weight of 0 8–1 0 g per day in young rats

1 Cornell Unit of riboflavin = 1 $\gamma$ of riboflavin defined by the growth effect on chicks [164]
1 B S Unit = 2 0–3 0 $\gamma$ of riboflavin [165, 166]
1 g riboflavin = 400 000–500 000 B S Units

## 13 Physiology of Plants and Microorganisms

The physiology of plants and microorganisms with respect to the action of riboflavin in their cells has not been studied sufficiently It is however reasonable to assume that principally the same reactions are carried out by riboflavin in plants and in microorganisms as in animals, especially as part of enzyme systems

Most microorganisms, bacteria [167] molds [168] fungi [169] etc , synthesize riboflavin, just as the higher plants do *Microbacillus tuberculosis*, for example, produces 0 5 to 2 9 $\gamma$ of riboflavin per day [170] A few, however have been found, for example special strains of lactic acid bacteria[171] and of the streptococci[172] which require an outside supply of riboflavin and are thus parasitic in character (see page 193)

In anaerobic cells for example in yeast in lactic and butyric acid bacteria, the riboflavin containing enzyme systems maintain the respiration[173] or that part of the respiration which cannot be disturbed by either hydrocyanic acid or by carbon monoxide and which therefore is not a function of a porphyrin enzyme system which contains iron and is easily poisoned Aerobic living bacteria contain and apparently need considerably less riboflavin than do the anaerobic living organisms

Riboflavin is as far as is known, synthesized by all higher plants It is apparently formed in the green leaves where it is found predomi

[163] H v Fuler In t tut intern tional de Ch m e Solvay Si eme Conseil de Ch m e rapport et discussions s r *Les V tam nes et l s Ho m e* P ris 1938 p 198
[164] L C Norris H S Wilgus A T R ngrose V He m n an l C 1 He ter *Cor ell Univ Ag I x ll Sta B ll* 660 3 (1936)
[165] O A Bessey *J Nut it on* 15 11 (1938)
[166] H v Euler P Karrer F Adler and M M lmberg *Helv Chim Acta* 17 11 7 (1934)
[167] O W rburg a d W Ch i ti n *Biochem Z* 266 377 (1933)
[168] J La ollay and F Laborey *Compt rend* 204 1686 (1937) 205 179 (1937) 06 1055 (1938)
[169] A Cu ll rmo d M Fontaine and A R ffy *Ib d* 201 1077 (1935) A Raffy *Ib d* 209 900 (1939)
[170] C. H Boisseva n W F Dren and H W Schultz *Pr c Soc Exptl Biol Med* 39 481 (1938)
[171] F B Snell a d F M Stro g *Enzym log a* 6 186 (1939)
[172] F J Kre sk pf P E Snell nd R McCoy *Ibid* 7 377 (1939)
[173] M Doudroff *Ibid* 5 239 (1938)

nantly In broccoli, the flower buds contain only a little over half as much as the leaves and the twigs contain even less [174] In carrots the riboflavin content of the roots is only one fourth of the content of the tops It has been shown that excised cosmos roots need a supply of vitamin $B_2$[175] which apparently is furnished in the intact plant from some other part, probably the leaves A beneficial effect on the growth of eggplants in synthetic nutrient solution has been observed upon the addition of 2 5% of riboflavin [176] There seems to exist a definite species difference in the response of various plants to riboflavin administration

As leaves become older, the riboflavin content diminishes Younger parts of plants always seem to contain more than older parts Ungerminated seeds generally contain little riboflavin (peas are an exception) During germination the riboflavin content increases many times

It has been reported that riboflavin increases the phototropic action of plants [177]

## 14 Animal Physiology

### (a) *General Physiology, Metabolism and Mechanism of the Vitamin $B_2$ Action*

The chemically bound vitamin $B_2$, as it occurs, for example in vegetables and in seeds, cannot be absorbed in the intestines of animals as has been shown in the case of the rat [178] After cooking, however, the vitamin is liberated and completely absorbed

The three naturally occurring free riboflavin compounds namely, riboflavin, riboflavin 5′ phosphoric acid and riboflavin adenine dinucleotide are easily absorbed by the intestines in the small gut The free riboflavin is phosphorylated in the intestines as can be shown *in vitro* with dried powdered intestinal mucosa or with glycerol extracts of intestines When the phosphorylation mechanism is disturbed, for example, experimentally with iodo acetic acid or by extirpation of the adrenals [179] riboflavin is not absorbed and the organism (rats were used for these experiments) ceases growing Only by the addition of riboflavin phosphoric acid (and possibly also of the dinucleotide) to the diet does further growth occur

[174] Mentioned in the review by H C Sherman and C S Lanford in *The Vitamins* Am Med Assoc 1939 p 292

[175] J Bonner *Am Chem Soc Div Agr Food Chem Meeting* Sept 1939 Abst 13–14

[176] R Dennison *Science* 92 17 (1940)

[177] M Heiman *Wien klin Wochschr* 49 398 (1936)

[178] F M Lantz *Agr Exptl Sta New Mexico Coll Agr Mech Arts Bull* 268 (1939)

[179] F Verzár and L Laszt *Arch ges Physiol (Pflügers)* 236 693 (1935) 237 476 483 (1936) *Verhandl Schweiz Physiol* I VI (1936) *Enzymologia* 3 16 (1937)

The transformation of riboflavin to its phosphoric acid ester and the dinucleotide is also a general cellular reaction Human blood cells, for example but not the plasma, can synthesize the dinucleotide from riboflavin both *in vitro* and *in vivo*[180] Therefore, riboflavin can also be administered parenterally

Riboflavin phosphoric acid is built into various enzyme systems which have been discussed previously The liver and kidney seem to undertake these reactions to a greater extent than the other organs

Riboflavin is excreted predominantly in the feces, and to a smaller extent in urine, During vitamin $B_2$ avitaminosis, no riboflavin is excreted in the urine small amounts however are found in the feces Vitamin $B_2$ is excreted mainly in the free form but in varying amounts, up to 50%, also as phosphoric acid ester An increase in the riboflavin intake of humans increases the urinary output of riboflavin[180, 181] The intake of riboflavin phosphoric acid increases to a small extent the excretion of the phosphorylated compound besides increasing to an appreciable extent the excretion of free riboflavin[182] Normal human beings on a balanced diet excrete about 500-800 γ per day[183]

Besides riboflavin and its phosphoric acid ester another flavin called aquoflavin or uroflavin is found in urine The chemical constitution of this compound is not quite clear it seems to contain more oxygen than does riboflavin and is more water soluble It is also sensitive to illumination and appears to be similarly converted into a lumiflavin compound[184] Uroflavin is apparently a degradation product of riboflavin

The animal organism has no special storage organs for riboflavin, although a certain level is maintained in the various tissues (0 5 γ per gram of blood[183]) The flavin dinucleotide level in blood cells and plasma is fairly constant[180] Relatively large amounts are found for example in liver and in kidney Intake of large amounts of riboflavin does not increase the riboflavin content of the liver to any appreciable extent[185] but increases the dinucleotide concentration in the blood cells[180] On the other hand, the organs of animals which die of vitamin $B_2$ avitaminosis still contain considerable amounts of this vitamin approximately one third of the normal level[185, 186] In man during times of clinical $B_2$ avitaminosis

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[180] J R Klein and H I Kohn *J Biol Chem* 136 177 (1940)

[181] A Emmerie and M van Eekelen *Acta Brev Neerland Physiol Pharmacol Microbiol* 7 109 (1937)

[182] A Emmerie *Ibid* 8 116 (1939)

[183] F M Strong R E Feeney B Moore and H T Parsons *J Biol Chem* 137 363 (1941)

[184] W Koschara *Z physiol Chem* 229 103 (1934) 232 101 (1935)

[185] R Kuhn H Kaltschmitt and T Wagner Jauregg *Ibid* 232 36 (1935)

[186] F Vi anco *Arkiv Kem Mineral Geol* A12 No 3 (1935)

no substantial decrease of the riboflavin content in blood and in muscles could be observed.[187] In rats[188] and in dogs,[189] riboflavin deficiency causes a decrease of the tissue level of this vitamin. The amount of the riboflavin adenine dinucleotide also decreases during times of low riboflavin intake and increases again upon administration of the vitamin (experiments with rats[190]). Thus, the xanthine oxidase activity in the livers of riboflavin depleted rats is only a small percentage of the activity in rats on an adequate riboflavin intake, as measured by the rate of oxygen consumption.[191]

Riboflavin is like all the other vitamins secreted in the milk, where it is found predominantly in the free form. In human milk, riboflavin is said to occur also to a small extent in combination with a protein.[192]

The fundamental action of riboflavin in living tissue is to take part in enzyme systems which regulate cellular oxidations. These enzyme systems have already been described. They take part in the general carbohydrate metabolism (fermentation and glycolysis). Riboflavin is also involved in the absorption of carbohydrates from the intestines by phosphorylation. Thus glucose and galactose are rapidly absorbed only in the presence of riboflavin.[193] *Riboflavin is connected to a certain extent with* the fat metabolism.[194] Increased fat content of the livers has been observed in hens and dogs which died of riboflavin insufficiency.[195, 196] Furthermore, it has been demonstrated[197] that the symptoms of a vitamin $B_2$ deficiency in rats increase in severity upon administration of increased amounts of fats. On the other hand, when increased doses of riboflavin are given together with increased amounts of fat no deleterious effect is observed. Riboflavin bears an important relation to the amino acid metabolism since *d* amino-acids are deaminated by an enzyme system, which contains the flavin adenine dinucleotide.

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187 A. F. Axelrod, T. D. Spies and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* 46, 146 (1941).

188 I. Vivanco, *Naturwissenschaften* 23, 306 (1935). R. Kuhn, H. Kaltschmitt and T. Wagner-Jauregg, *Z. physiol. Chem.* 232, 36 (1935). J. Croen and J. W. Schuyl, *Arch. néerland. physiol.* 23, 271 (1938).

189 H. F. Frazer, N. H. Topping and H. Isbell, *U. S. Pub. Health Service Pub. Health Repts.* 55, 80 (1940).

190 S. Ochoa and R. J. Rossiter, *Biochem. J.* 33, 2008 (1939).

191 A. E. Axelrod and C. A. Elvehjem, *Proc. Am. Soc. Biol. Chem.* 1941, VI.

192 P. Ellinger and W. Koschara, *Nature* 133, 553 (1934).

193 L. Laszt and F. Verzár, *Biochem. Z.* 292, 159 (193 ). M. J. Iowitz and F. Verzár, *Biochem. J.* 292, 18? (1937).

194 E. W. McHenry and G. Gavin, *J. Biol. Chem.* 125, 6 3 (1938).

195 S. Lepkovsky, L. W. Taylor, T. H. Jukes and H. J. Almquist, *Hilgardia* 11, ?59 (1938).

196 W. H. Sebrell and R. H. Onstott, *U. S. Pub. Health Service Pub. Health Repts.* 53, 83 (1938). H. R. Street and G. R. Cowgill, *Am. J. Physiol.* 125, 323 (1939).

197 G. J. Mannering, M. A. Lipton and C. A. Elvehjem, *Proc. Soc. Exptl. Biol. Med.* 46, 100 (1941).

Riboflavin in the free form plays an important role in the vision mechanism in the retina [198] Light converts riboflavin into a 'photo compound of unknown structure, which process seems to have some bearing on the stimulation mechanism of the optical nerve The primary 'photo compound' is extremely sensitive In the absence of oxygen it is destroyed in the presence of oxygen it is reconverted into riboflavin (Theorell) The mechanism of the riboflavin action in the retina is especially well understood for dim light since light of short wave length is converted into light of longer (yellow green) waves by the fluorescent activity of riboflavin [199] The human eye has a maximum sensitivity for greenish light

The theory has also been advanced that riboflavin takes part in an oxidation system in the cornea [200] Since the cornea is avascular, the cornea cells are nourished according to this hypothesis by a specific riboflavin containing enzyme system It is thought that during riboflavin deficiency the body attempts to counteract the missing oxygenation by vascularization

An interesting fact about riboflavin is that the phosphorescence of the glow worm (*Lampyris*) is caused by riboflavin in combination with a special protein [201] The phosphorescence consists of light of the wave lengths 562–570 mμ It must be assumed that riboflavin plays an important role in some unknown biochemical process in the luminous organs

It is also interesting that the mold *Aspergillus niger* which is free of riboflavin when cultivated under optimum conditions becomes tinted with riboflavin on a medium deficient in magnesium The formation of the pigment is not obtained by restricting the other elements but is increased if a deficiency in magnesium and iron exists simultaneously [202]

### (b) *Relation of Vitamin B to Other Vitamins Hormones and Minerals*

Riboflavin bears an obvious relation to other members of the vitamin B group It seems that most of these vitamins act as part of enzyme systems which regulate the carbohydrate fat and amino acid metabolism For example the close relation of riboflavin to the enzyme systems containing nicotinic amide has already been discussed The relation of vita

[198] A M Chase *Science* 85 484 (1937)

[199] H v Euler and F Adler *Ark v Kem Mineral Geol* B11 No 78 (1934) R Kuhn and H Kaltschmitt *Ber* 68 386 (1935) F Adler and H v Euler *Nature* 141 790 (1938) H v Euler and F Adler *Z physiol Chem* 223 105 (1934)

[200] O A Bessey and S B Wolbach *J Exptl Med* 69 1 (1939) R F Eckardt and L V Johnson *Arch Ophthalmol* 21 315 (1939)

[201] C Brook *Compt rend* 210 798 (1940)

[202] J Lavollay and F Laborey *Ibid* 208, 1056 (1939)

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[187] A E Axelrod T D Spies and C A Elvehjem *Proc Soc Exptl Biol Med* 46 146 (1941)

[188] F Vivanco *Naturwissenschaften* 23 306 (1935) R Kuhn H Kaltschmitt and T Wagner Jauregg *Z physiol Chem* 232 36 (1935) J Croen and J W Schuyl *Arch néerland physiol* 23 271 (1938)

[189] H F Frazer N H Topping and H Isbell *U S Pub Health Service Pub Health Repts* 55 80 (1940)

[190] S Ochoa and R J Rossiter *Biochem J* 33 2008 (1939)

[191] A E Axelrod and C A Elvehjem *Proc Am Soc Biol Chem* 1941 VI

[192] P Ellinger and W Koschara *Nature* 133 553 (1934)

[193] L Laszt and F Verzár *Biochem Z* 292 159 (1937) M Jurlowitz and F Verzár *Biochem Z* 292 182 (1937)

[194] F W McHenry and G Gavin *J Biol Chem* 125 653 (1938)

[195] S Lepkovsky L W Taylor T H Jukes and H J Almquist *Hilgardia* 11 559 (1938)

[196] W H Sebrell and R H Onstott *U S Pub Health Service Pub Health Repts* 53 83 (1938) H R Street and G R Cowgill *Am J Physiol* 125 323 (1939)

[197] G J Mannering M A Lipton and C A Elvehjem *Proc Soc Exptl Biol Med* 46 100 (1941)

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[198] A M Chase *Science* 85 484 (1937)

[199] H v Euler and E Adler *Ark v Kemi Mineral Geol* B11 No 28 (1934) R Kuhn and H Kaltschmitt *Ber* 68 386 (1935) F Adler and H v Eul r *Nature* 141 790 (1938) H v E ler and F Adler *Z phy ol Chem* 223 105 (1934)

[200] O A Bessey and S B Wolb h *J Expil Med* 69 1 (1939) R F Eck rdt and L V Johnson *A h Ophthalmol* 21 315 (1939)

[201] G Brooks *Compt re d* 210 2 8 (1940)

[202] J Lavoll y and F Labor y *Ibid* 208 10 6 (1939)

min $B_2$ to vitamin $B_1$ may be demonstrated by the fact that riboflavin has an apparent sparing action on thiamin[203]

An important relation exists between riboflavin and the adrenal cortex hormone the phosphorylation of riboflavin is carried out with the aid of the adrenal cortex hormone Adrenalectomized animals lose their power of phosphorylating riboflavin Thus, riboflavin phosphoric acid, but not riboflavin, maintains life and growth of adrenalectomized rats[204]

A number of observations have been made which demonstrate the close relationship of riboflavin to other hormones concerned in the carbohydrate metabolism Rats lose their liver glycogen after injections of thyroxine unless increased amounts of thiamine and riboflavin are given simultaneously[20] Depancreatized dogs respond to insulin only in the presence of thiamin and riboflavin, but not in the presence of one of these vitamins alone[206]

A certain relation of riboflavin to the minimum amount of indispensable magnesium is noted A minimum riboflavin intake increases the minimum magnesium requirements[207]

## 15 Avitaminosis and Hypovitaminosis

The basic but unspecific symptoms of a riboflavin deficiency are the same throughout the entire animal kingdom—cessation in growth of young organisms and sudden death of adult organisms

In rats, riboflavin deficiency, even in a relatively early stage, considerably decreases the resistance forces of the organism against infectious diseases for example endemic typhus[08] Growth of young rats is impaired when fed a vitamin $B_2$ deficient diet and when no riboflavin is administered the animals die Riboflavin deficient animals sometimes show an abnormal intracellular metabolism[208] In riboflavin deficient rats an early atrophy of the testis and an involution of the thymus have been observed[209] Increase of the diaphragm metabolism in rats is also reported[210] The development of alopecia (baldness) and cataract (loss of transparency of the

[204] L N Ellis and A Zmachinsky *Science* 86 245 (1937)

[20] F Verzár and L Laszt *Arch ges Physiol (Pflügers)* 236 693 (193?) 237 476 483 (1936) *Verhandl Schweiz Physiol* I VI (1936) *Enzymologia* 3 16 (1937)

[20] V A Drill *J Nutrition* 14 355 (1937)

[206] R W Martin *Z physiol Chem* 248 242 (1937) *Verhandl deut Ges inn Med* 50 4?0 (1938)

[207] E V Tufs and D M Greenberg *J Biol Chem* 122 715 (1938) *Am J Physiol* 121 416 (1938)

[208] H Pinkerton and O A Bessey *Science* 89 369 (1939)

[209] J W Schuyl and J Croen *Acta Brevia Neerland Physiol Pharmacol Microbiol* 8 195 (1938) J H Shaw and P H Phillips *J Nutrition* 22 345 (1941)

[210] A B Hastings J Muus and O A Bessey *J Biol Chem* 129 295 (193?)

lens of eyes) in rats on riboflavin deficient diets is of special interest [211, 212] Certain eye lesions namely, diffuse corneal opacity and vascularization of the cornea, are specific for riboflavin deficiency in rats [13, 214]

Pigs also need riboflavin for normal growth and physical well being [215] Dogs usually collapse after about 100 days on a riboflavin deficient diet [216] For chicks and fowls riboflavin is of importance in the production of eggs and for normal hatchability of eggs [217, 218, 219, 220] Chicks hatched from eggs on a partially riboflavin deficient ration exhibit a characteristic curled toe ( nutritional leg paralysis ) [220] Besides this slowly developing paralysis an acute paralysis characterized as neuromalacia was observed [221] The main peripheral nerve trunks are especially involved and characteristic changes in the myelin have been noticed These severe nerve degenerations occur also in rats [222] Turkey poults develop a typical dermatitis as the result of a riboflavin deficiency [223] In addition to the specific symptoms which are caused by a riboflavin deficiency a premature aging has been observed in all animals studied [224]

Riboflavin deficiency in humans of all ages[225] causes the occurrence of specific symptoms Primarily cheilosis an eversion of the lips and in the corners of the mouth has been observed [6, 227] There is also a seborrheic syndrome, a scaly slightly greasy desquamation on a mildly erythematous base in the nasobial fold on the alae nasi in the vestibule of the nose and on the ears A special type of glossitis may develop which is characterized by a purplish red or magenta colored inflammation of the tongue [228] The entire condition is called ariboflavinosis [226]

Riboflavin deficiency in man also causes characteristic ocular symptoms similar to those already described for rats In mild cases a sensation of

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[211] P L Day W C Langston and C S O Brien *Am J Opthalmol* 14 100 (1931)
[212] P L Day W J Darby and W C Langston *J Nutrition* 13 389 (1937)
[213] M M El Sadr *J Soc Chem Ind* 58 1070 (1939)
[214] O A Bessey and S B Wolbach *J Exptl Med* 69 1 (1939) R E Eckardt and L V Johnson *Arch Opthalmol* 21 315 (1939)
[215] E H Hughes *J Nutrition* 17 527 (1939)
[216] H R Street and G R Cowgill *Am J Physiol* 125 323 (1939)
[217] S Lepkovsky L W Taylor T H Jukes and H J Almquist *Hilgardia* 11 [illegible] (1938)
[218] H J Davis L C Norris and G F Heuser *Poultry Sci* 17 81 87 (1938)
[219] G F Heuser H S Wilgus and L C Norris *Ibid* 17 105 (1938)
[220] A E Schumacher and G F Heuser *Ibid* 18 369 (1939)
[221] P H Phillips and R W Engel *J Nutrition* 16 451 (1938) *Poultry Sci* 17 413 (1938) E L R Stokstad and F D V Manning *J Nutrition* 16 279 (1938)
[222] J H Shaw and P H Phillips *J Nutrition* 22 345 (1941)
[223] S Lepkovsky and T H Jukes *J Nutrition* 12 515 (1938)
[224] P L Day and W J Darby *J Biol Chem* 123 8 (1938)
[225] T D Spies W B Bean R W Vilter and N E Huff *Am J Med Sci* 200 697 (1940)
[226] W H Sebrell and R E Butler *U S Pub Health Service Pub Health Repts* 53 2282 (1938)
[227] J W Oden L H Oden and W H Sebrell *Ibid* 54 790 (1939)
[228] H D Kruse V P Sydenstricker W H Sebrell and H M Cleckley *Ibid* 55 157 (1940)

roughness in the eyes and itching and burning are observed[229] which are accompanied by a mild photophobia In more severe cases a corneal opacity has been noted [228] Interstitial keratitis (of the cornea) in cases of syphilis improves markedly upon treatment with riboflavin [28] Persons showing subnormal dark adaptation which does not respond to vitamin A treatment may be relieved by riboflavin administration [230, 231]

Riboflavin treatment is also of importance in diseases caused by multiple vitamin deficiencies, such as pellagra,[232] black tongue[233] and beriberi Beneficial results from riboflavin have also been observed in a case of pemphigus [234]

## (a) *Clinical Test Methods*

Vitamin $B_2$ can be determined in blood, in muscles and in urine The value of these tests for a detection of a state of a hypovitaminosis or avitaminosis is, however, very limited While the riboflavin content of tissues from experimental animals, especially from rats, is lowered during times of inadequate vitamin $B_2$ intake [235, 236, 237, 238, 239, 240] this is not the case in human beings as is evident from blood and muscle analysis [241] The most promising test is the tissue saturation test but not enough experimental data have accumulated to determine the actual value of this procedure *Blood determinations* for riboflavin are best carried out by the Lactobacillus test,[242] the reliability of which has been established [243, 244] The riboflavin adenine dinucleotide content of blood can be determined by the *d* amino acid oxidase test [245] In *muscle studies* the quadriceps femoris muscle[241] and the Lactobacillus test[242] are used for the determination of the riboflavin

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[229] T D Spies R W Vilter and W F Ashe *J Am Med Assoc* 113 931 (1939)
[230] M S Kimble and E S Gordon *J Biol Chem* 128 lii (1939)
[231] P H Pock Steen *Aknephascopia Geneesk tijschr v Nederl Indie* 79 1986 (1939)
[232] R W Vilter S Vilter and T D Spies *J Am Med Assoc* 112 420 (1939)
[233] I H Margolis G Margolis and S G Smith *J Nutrition* 17 63 (1939)
[234] M C Topping and A F Knoefel *J Am Med Assoc* 114 210 (1940)
[235] S Ochoa and R J Rossiter *Biochem J* 33 008 (1939)
[236] A E Axelrod H A Sober and C A Elvehjem *J Biol Chem* 134 749 (1940)
[237] R Kuhn H Kaltschmitt and T Wagner Jauregg *Z physiol Chem* 232 36 (1935)
[238] J Groen and J W Schuyl *Arch néerland physiol* 23 271 (1938)
[239] E V Carlsson and H C Sherman *J Nutrition* 15 57 (1938)
[240] H F Frazer N H Topping and H Isbell *U S Pub Health Service Pub Health Repts* 55 280 (1940)
[241] A E Axelrod T D Spies and C A Elvehjem *Proc Soc Exptl Biol Med* 46 146 (1941)
[242] E F Snell and F M Strong *Ind Eng Chem Anal Ed* 11 346 (1939) *Enzymologia* 6 186 (1939) E E Snell F M Strong and W H Peterson *Biochem J* 31 1789 (1937) *J Am Chem Soc* 60 2825 (1938)
[243] R E Feeney and F M Strong *Proc Am Soc Biol Chem* 1940 XXXI
[244] F M Strong R E Feeney B Moore and H T Parson *J Biol Chem* 137 363 (1941)
[245] J R Klein and H I Kohn *Ibid* 136 177 (1940)

present The amount of riboflavin present *in urine* can be assayed by the Lactobacillus test[244] or by the fluorescence method Urine containing 0 0001 mg or less of riboflavin per cc cannot be used in the latter procedure [245] In the *tissue saturation test* the amount of riboflavin excreted through the urine is measured after oral administration of excess doses of the vitamin [245]

## 16 Hypervitaminosis

Riboflavin administered in excessive amounts by mouth was found to be non toxic in dogs and rats Following intraperitoneal injection however riboflavin produced death due to kidney concretions [246, 247]

## 17 Requirements

A regular dietary intake of riboflavin is necessary for all members of the animal kingdom and for some microorganisms From eleven species of lactic acid bacteria investigated, four were found to require riboflavin for growth and acid production The remaining seven did not require an external supply of this vitamin and it has been established that four of these seven species synthesize vitamin B when cultured on a riboflavin free medium [248, 249] Riboflavin also stimulates the growth of propionic acid [250] and of luminous[251] bacteria

The only organism so far reported as actually needing the riboflavin adenine dinucleotide is the larva of mosquitoes Riboflavin itself is not effective [252]

In general, the vitamin $B_2$ requirements of animals are related to body size and weight, to the amount of food ingested to the ambient temperature etc Man needs about 2–3 mg of riboflavin daily [253, 254] The riboflavin allowances recommended by the Food and Nutrition Board National Research Council, will be found on page 613

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[244] R Kuhn and P Boulanger *Z physiol Chem* 241 233 (1936) R Kuhn *Klin Wochschr* 17 22 (1938) V Demole *Z Vitamins forsch* 7 138 (1938)

[245] K Unna and J G Greslin *J Pharmacol* 76 75 (1942)

E E Snell and F M Strong *Enzymologia* 6 186 (1939)

[248] E E Snell F M Strong and W H Peterson *Biochem J* 31 1789 (1937) S Orla Jensen N C Otte and A Snog Kjaer *Zentr Bakt Parasitenk* II 94 434 447 (1936) H G Wood A A Anderson and C H Werkman *J Bact* 34 132 (1937) *Proc Soc Exptl Biol Med* 36 217 (1937)

[250] H G Wood A A Anderson and C H Werkman *J Bact* 36 201 (1938)

[251] M Doudoroff *Enzymologia* 5 239 (1938)

[252] W Trager and Y Subbarow *Biol Bull* 75 7 (1938)

[253] W Stepp J Kühn u a d H Schroeder *Die Vitamine* Stuttgart 1937 p 7

[254] W H Sebrell R E Butler J C Wooley and H Isbell *U S Pub Health Service Pub Health Repts* 56 510 (1941)

Chickens need 230–245 $\gamma$ per 100 g of diet to maintain normal hatchability [55, 256] Baby chicks need increased amounts [256] Turkeys require about 25% more riboflavin than chickens

Some animals apparently need no riboflavin or only a very small amount in their food Microorganisms which synthesize this vitamin live in the rumen of sheep[257] and of cattle[258, 259] and make the vitamin available to the animal

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[255] H J Davis L C Norris and G F Heuser *Poultry Sci* 17 81 87 (1938)
[256] G F Heuser H S Wilgus and L C Norris *Ibid* 17 105 (1938)
[257] L W McElroy and H Goss *J Biol Chem* 130 437 (1939)
[258] L W McElroy and H Goss *Ibid* 133 LXV (1940)
[259] M I Wegner A N Booth C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 45 769 (1940)

# VITAMIN $B_6$—PYRIDOXIN

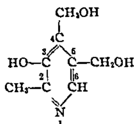
# VITAMIN $B_6$—PYRIDOXIN

## 1 Nomenclature and Survey

**Names**

Pyridoxin Term suggested by Gyorgy[1] and generally adopted in the United States
Adermin European name
Vitamin $B_6$ of Gyorgy
Anti acrodynia rat factor
Rat antidermatitis factor
Yeast eluate factor[2,3]
Factor 1 of Lepkovsky[4]
Factor Y of Chick[5]
Vitamin H of Richardson and Hogan[6] and of Booher[7,7]
Complimentary factor[8]

**Chemical formula**




**Chemical name**

3 Hydroxy-4 5-di(hydroxy methyl) 2 methyl pyridine

**Empirical formula**

$C\ H_{11}O_3N$

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[1] P György and R L I ckhardt *Nature* 144 ɔ12 (1939)
[2] C E Edgar and T F Macrae *B ochem J* 31 886 (1937)
M M El S dr T F Macrae and G E Work *Ibid* 33 611 (1939)
S Lepko sky T H Jukes and M E Kr use *J Biol Chem* 115 557 (1936)
[5] H Chick A M Copp ng and M H Roscoe *Biochem J* 24 1748 (1930)
L R Richardson and A G H gan *Missouri Agr Exptl Station Resea ch Bull* No 241 (1936) *Sc nce* 83 17 (1936)
[7] L I Booher *J Biol Chem* 119 2?3 (1937)
The term v tam n H was u ed n earl r days of vitam n re earch to designate vitamin B [9] Th a ne letter H has also b en used for a trout growth factor [10] In p esent day l terature the term v tam n H s reserved for b ot n the cur tive factor for egg white injury (see page 469)
[8] P György R Kuhn and T Wagner Jauregg *Natu wissenschaften* 21 561 (1933) *Klin Woch chr* 12 1241 (1933)
[9] A C Hogan n *The Vitam s* Am Med Assoc Ch c go 1939 p 273
[10] C M McCay F C B ng and W E D ll y *Science* 67 249 (19 8)

## 2 Chronology

1926 GOLDBERGER and LILLIE[11] reported the occurrence of a characteristic dermatitis called acrodynia on rats fed a diet deficient in vitamin $B_2$

1932 OHDAKE[12] in Japan isolated a compound of the formula $C_8H_{11}O_3N$ HCl from rice polishings but failed to recognize its vitamin character

1934 GYÖRGY[13] established the difference of the rat pellagra preventive factor from vitamin $B_2$ (and vitamin $B_4$) and called the new vitamin $B_6$

1938 The isolation of the pure crystalline vitamin $B_6$ was announced independently by five different groups namely by LEPKOVSKY[14] KERESZTESY and STEVENS,[15] GYÖRGY[16] KUHN and WENDT[17] and ITIBA and MITI[18]

1939 The chemical structure was elucidated and vitamin $B_6$ was synthesized independently by two groups of workers—by KUHN WESTPHAL WENDT and WESTPHAL in Germany and by KERESZTESY STEVENS HARRIS STILLER and FOLKERS in the U S A

## 3 Occurrence

Vitamin $B_6$ appears to be very widely distributed over the entire animal and plant kingdom Reliable systematic studies as to the relative quantities in various foodstuffs are scarce Yeast and rice polishings are especially rich in vitamin $B_6$ Seeds and cereals, for example, wheat and maize, are good sources[19] especially the germs and the integuments[20] Molasses[21] fish and fish livers[22] and mammaliar livers contain moderate amounts[21] [23] and milk egg yolks, lettuce, spinach, etc, contain small amounts of vitamin $B_6$

Vitamin $B_6$ occurs in animal and in plant tissues, for example, in yeast[24] and in fish muscle,[25] only to a small extent in the free form The majority (60 80%) is chemically bound to protein[4] and to starch[26]

[11] J Goldberger and R D Lillie *U S Pub Health Service Pub Health Rept* 41 1025 (1926)
[12] S Ohdake *Bull Agr Chem Soc Japan* 8 111 (1932) P W Wiardi *Nature* 142 1158 (1938)
[13] P György *Nature* 133 498 (1934) *Biochem J* 29 741 760 767 (1935)
[14] S Lepkovsky *Science* 87 169 (1938)
[15] J C Keresztesy and J R Stevens *Proc Soc Exptl Biol Med* 38 64 (1938)
[16] P György *J Am Chem Soc* 60 983 (1938)
[17] R Kuhn and G Wendt *Ber* 71 780 1118 (1938)
[18] A Itiba and K Miti *Sci Papers Inst Phys Chem Research (Tokyo)* 34 623 (1938)
[19] H A Schneider J K Ascham B R Platz and H Steenbock *J Nutrition* 18 99 (1939)
[20] A M Copping *Biochem J* 30 849 (1936)
[21] A van Schoor *Merck s Jahresberichte* 52 7 (1938)
[22] G Lunde and H Kringstad *Biochem J* 32 708 (1938)
[23] C E Edgar M M El Sadr and T F Macrae *Ibid* 32 2225 (1938)
[24] R Kuhn and G Wendt *Ber* 71 780 (1938)
[25] T W Birch and P György *Biochem J* 30 304 (1936)
[26] H Chick M M El Sadr and A N Worden *Biochem J* 34 595 (1940)

## 4 Isolation

As has been pointed out in the previous section vitamin $B_6$ occurs to a great extent bound to a protein From this symplex, vitamin $B_6$ cannot be separated by dialysis The symplex can however be split by heating[27, 28, 29] or by enzymatic hydrolysis [30]

The free vitamin $B_6$ is extracted with water or with organic solvents such as ether, propanol or butanol [31, 32] The latter solvents extract less by products than does water, but continuous extraction is necessary due to the water solubility of the vitamin

From neutral or acidified water solutions, vitamin $B_6$ can be adsorbed on charcoal and fuller s earth [33] The adsorption on fuller s earth is greatly influenced by the pH of the solution At pH 5–6 vitamin $B_6$ is not quantitatively adsorbed even after three consecutive adsorptions At pH 1 factors other than $B_6$ are also adsorbed [34] The elution is carried out with barium hydroxide[35] or with butyl alcohol [36] Vitamin $B_6$ is quantitatively adsorbed on zeolite and can be subsequently eluted with 10% potassium chloride [37] Inert material can be removed with acetone ethyl alcohol ethyl acetate platinum chloride etc The vitamin is precipitated by a number of acids, such as by sulfuric acid, phosphotungstic acid, silicotungstic acid Reinecke's acid etc By repeated precipitations the pure vitamin $B_6$ can be obtained in the form of various salts, such as the hydrochloride The free base is prepared therefrom by treatment with silver salts [38]

## 5 Properties

Vitamin $B_6$, as a free base, is a colorless crystalline powder has a slightly bitter taste and melts at 160° C [38, 39] It is readily soluble in water, in acetone and in alcohol, and slightly soluble in ether and chloroform Vitamin $B_6$ dialyzes easily It crystallizes in the form of various salts

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[27] R Kuhn and G Wendt *Ber* 71 780 (1938)

[28] E M Lantz *New Mexico Ag Exptl Station Bull* No 268

[29] H Chick M M El Sadr and A N Worden *Biochem J* 34 5?? (1940)

[30] T W Birch and P György *Ibid* 30 304 (1936)

[31] J V Scudi H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)

[32] R D Greene *J Biol Chem* 130 513 (1939)

[33] T W Birch and P György *Biochem J* 30 304 (1936)

[34] G Lunde and H Kringstad *J Nutrition* 19 321 (1940)

[35] N Halliday and H M Evans *J Biol Chem* 118 ?55 (1937)

[36] G A Emerson A Mohammad O H Emerson and H M Evans *Ibid* 124 377 (1938)

[37] J V Scudi H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)

[38] A Ichiba and K Michi *Sci Papers Inst Phys Chem Research* (*Tokyo*) 34 1014 (1938)

[39] J C. Keresztesy and J P Stevens *J Am Chem Soc* 60 1267 (1938)

for example as hydrochloride, m p 204–206° C [39] (with decomposition), and as picrate The hydrochloride is soluble in water (1 g in 4 5 cc water) and in alcohol (1 g in 90 cc alcohol) and somewhat soluble in acetone The aqueous solution has a pH of about 3 2 Both the free vitamin $B_6$ and its hydrochloride sublime readily [39] The hydrochloride is the form in which this vitamin is marketed It is a white odorless powder with salty taste It is stable to heat, concentrated hydrochloric acid and alkali, but is destroyed by light

Vitamin $B_6$ is optically inactive It exhibits a typical ultraviolet absorption spectrum which changes markedly with a change of hydrogen ion concentration The spectra of the vitamin in aqueous solution between pH 4 and 6 75 are shown in Fig 11

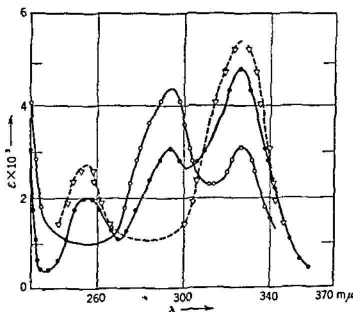



Fig 11 —Absorption spectra of vitamin $B_6$ at O pH 4 ● pH 5 1 ∇ pH 6 75 (E T Stiller J C Keresztesy and J R Stevens)

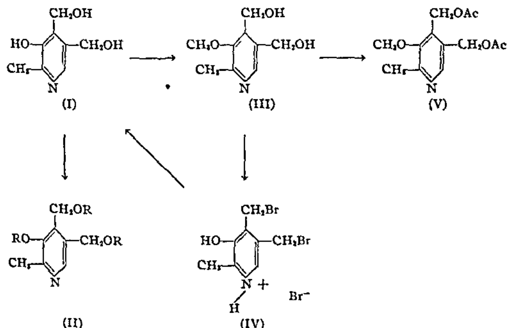
## 6 Constitution

Vitamin $B_6$ (I) has the empirical formula $C_8H_{11}O_3N$ and forms salts easily with acids, such as hydrochloric acid picric acid, etc The hydrochloride yields an orange red color with ferric chloride and couples with diazonium salts, for example with diazotized sulfanilic acid which properties suggest the presence of a phenolic or enolic hydroxyl group [40]

[40] R Kuhn and G Wendt *Ber* 71 1118 (1938)

All three oxygens of the molecule are present in the form of hydroxyl groups A triacetate which can be distilled [41, 42] and a tribenzoate[43] (II) can be prepared An estimation of active hydrogen atoms showed the presence of three in the molecule [43]

Upon reaction with diazomethane a compound, $C_9H_{13}O_3N$, is obtained which does not give a color reaction with ferric chloride and which does not couple with diazotized sulfanilic acid Treatment of this compound with hydriodic acid yields methyl iodide Quantitative estimation of these properties led to the conclusion that a mono methyl ether of vitamin $B_6$ (III) is produced by diazomethane [44] Vitamin $B_6$ methyl ether is split upon reaction with hydrobromic acid to a hydrobromide of a dibromide, $C_8H_{10}ONBr_3$ (IV), which yields vitamin $B_6$ by treatment with silver ace tate [45]




Besides the methyl ether, diazomethane produces by reaction with vitamin $B_6$ the *N* methyl vitamin $B_6$ [46] which yields a color reaction with ferric chloride

[41] R Kuhn and G Wendt *Be* 71 780 (1938)
[42] A Itiba and K M ti *Sci Papers In t Phys Chem R earch (T ky )* 35 73 (1938) 36 1 (1939)
[43] E T Still r J C Kere zt y a d J R Steven *J Am Chem Soc* 61 1237 (1939)
[44] R Kuhn and G Wendt *Ber* 71 1534 (1938)
[45] R Kuhn nd G Wendt *Ibid* 72 311 (1939)
[46] A Itiba and K Mit *Sci Papers Inst Phys Chem Reseu ch (T kyo)* 35 73 (1938) 36 1 (1939)

The methyl ether of vitamin $B_6$ is converted by the action of acetic anhydride in pyridine into a diacetyl methyl ether (V) Thus it is concluded that of the three hydroxyl groups one is phenolic (or enolic) and the other two are aliphatic hydroxyl groups All active hydrogen atoms are accounted for and no further active hydrogen atoms could be detected in the diacetyl methyl ether Therefore, it must be concluded that the nitrogen is present in a ring [47]

The position of the phenolic hydroxyl group was determined, first, by application of the Folin Denis phenol reagent[48] which produced a color with vitamin $B_6$ and with $\beta$ hydroxy pyridine, but not with $\alpha$ and $\gamma$ hydroxy pyridine,[49] and second, by investigation of the ultraviolet absorption spectrum which proved to be similar to that of $\beta$ hydroxy pyridine [49, 50] Upon application of the color test with 2,6 dichloro quinone chlorimide vitamin $B_6$ gives a blue color,[50] which effect proves, according to Gibbs,[51] that the $p$ position to the hydroxyl group is not substituted

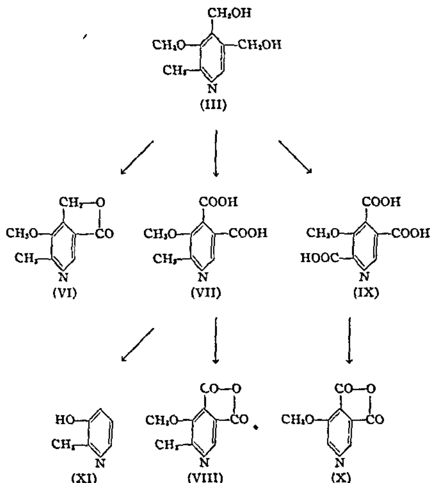
Vitamin $B_6$ methyl ether does not react with lead tetra acetate, which indicates that the two aliphatic hydroxyl groups are not in $\alpha$ $\beta$ position [49] Oxidation in neutral aqueous solution with potassium permanganate in an amount corresponding to two atoms of oxygen converts vitamin $B_6$ methyl ether (III) into a lactone (VI), which indicates that the two aliphatic hydroxyl groups are either in 1,4- or in 1 5 position to each other [49, 51] Upon further oxidation of vitamin $B_6$ methyl ether with barium permanganate, four atoms of oxygen are taken up The reaction product is a dicarboxylic acid (VII)[52, 53, 54, 55] which contains all the carbon atoms of the vitamin methyl ether By treatment of this dicarboxylic acid with acetic anhydride the dicarboxylic acid is dehydrated yielding the anhydride (VIII),[54] indicating that the two carboxyl groups are vicinal Indication of the same fact is given by the fusion of the dicarboxylic acid with resorcinol[54] which yielded a phthalein having a greenish yellow fluorescence Decarboxylation of the dibasic acid, by heating the disodium salt with calcium hydroxide, yielded a hydroxy picoline (XI) [55]

Vitamin $B_6$ methyl ether on oxidation with potassium permanganate in alkaline solution takes up seven atoms of oxygen and forms a tricarboxylic

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[47] R Kuhn and G Wendt *Ber* 71 1534 (1938)
[48] O Folin and W Denis *J Biol Chem* 12 239 (1912) 22 305 (1915)
[49] R Kuhn and G Wendt *Ber* 72 305 (1939)
[50] E T Stiller J C Keresztesy and J R Stevens *J Am Chem Soc* 61 1237 (1939)
[51] H D Gibbs *J Biol Chem* 72 649 (1927) E J Theriault *Ind Eng Chem* 21 343 (19 9)
[52] A Itiba and K Miti *Sci Papers Inst Phys Chem Research (Tokyo)* 35 73 (1938) 36 1 (1939)
[53] R Kuhn H Andersag K Westphal and G Wendt *Ber* 72 309 (1939)
[54] R Kuhn G Wendt and K Westphal *Ibid* 72 310 (1939)
[55] E T Stiller J C Keresztesy and J R Stevens *J Am Chem Soc* 61 1?37 (1939)

acid (IX) without the loss of carbon atoms [56] This tricarboxylic acid yields an anhydride of a dicarboxylic acid (X) while simultaneously losing one mol of carbon dioxide [56] The tricarboxylic acid yields with ferrous sulfate a reddish color that is characteristic for pyridine α-carboxylic acids




This color reaction is not given by the dicarboxylic acids (VII) and (X) This proves that the carboxyl group lost by anhydration of the tricarboxylic acid was in α position to the ring nitrogen[56] and that this carboxyl group originated from a methyl group [57] The existence of the methyl group has also been shown by oxidation of the vitamin chlorohydrate with chromic acid in sulfuric acid whereby acetic acid is obtained

[56] R Kuhn and C Wendt *Ber* 72 305 (1939)
[57] R Kuhn H Andersag K Westphal and G Wendt *Ibid* 72 309 (1939)

## 7 Synthesis

Two different methods have been described for the synthesis of vitamin $B_6$, one of which is a complete synthesis building up the pyridine nucleus from small aliphatic molecules The other synthesis is a partial degradation of a higher molecular compound to the pyridine derivative of the constitution of vitamin $B_6$

### (a) *The Complete Synthesis of Harris and Folkers*[58, 59] *and of Mori and Makino*[60]

By a series of seven reactions vitamin $B_6$ has been synthesized as follows

*Step 1* Cyano acetamide (I) is condensed with ethoxy acetyl acetone (II) in the presence of piperidine to yield 3 cyano 4 ethoxy methyl 6 methyl 2 pyridone (III)

*Step 2* By nitration of the reaction product of step *1*, 3 cyano 4 ethoxy methyl 5 nitro 6 methyl 2 pyridone (IV) is obtained

*Step 3* Chlorination converts the last mentioned compound (IV) into 2 methyl 3 nitro 4 ethoxy methyl 5 cyano 6 chloro pyridine (V)

*Step 4* Partial catalytic hydrogenation of (V) yields 2 methyl 3 amino-4 ethoxy methyl 5 cyano 6 chloro pyridine (VI)

By a less attractive series of reactions the amino chloro compound (VI) can be obtained from the nitro pyridone (IV) by reduction of the nitro group to the amino pyridone (X) followed by chlorination

*Step 5* The cyano chloro pyridine (VI) is catalytically hydrogenated to 2 methyl 3 amino 4 ethoxy methyl 5 amino methyl pyridine (VII)

A modification of this step consists in first acetylating the 3 amino group of (VI) to give (XI) followed by hydrogenation to remove the chlorine and to convert the cyano group in the amino methyl group (XII) Finally the acetyl groups of the 3 amino group are split off by hydrolysis

*Step 6* The 4 ethoxy group is now saponified with dilute hydrochloric acid to yield the dihydrochloride of 2 methyl 3 amino 4 hydroxy methyl 5 amino methyl pyridine (VIII)

If this reaction is carried out with hydrobromic acid, the dihydrobromide of a 4 bromo methyl compound (XIII) is obtained, which must be saponified to give (VIII)

*Step 7* The last step of this synthesis consists in diazotization of the diamine (VIII) to yield vitamin $B_6$ (IX)

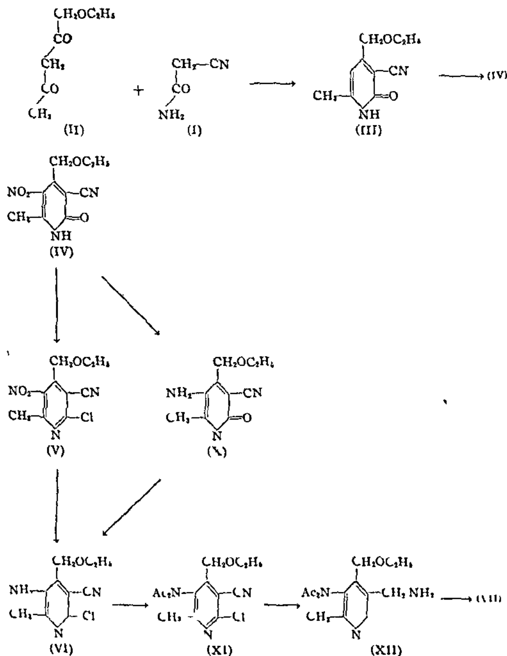
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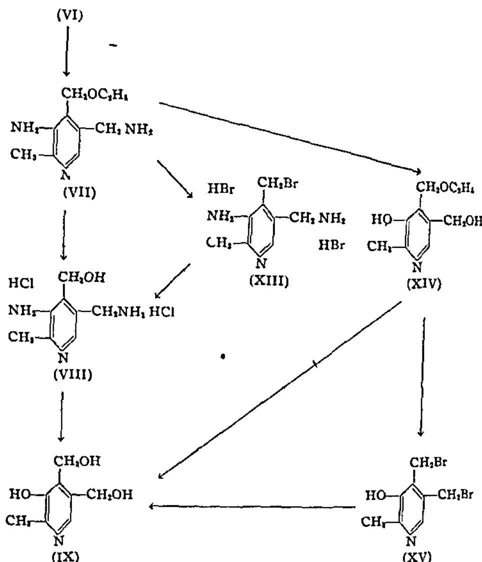
[58] S A Harris and K Folkers *J Am Chem Soc* 61 1245 (1939) S A Harris E T Stiller and K Folkers *Ibid* 61 1242 (1939)

[59] S A Harris and K Folkers *Ibid* 61 3307 (1939)

[60] S Mori and K Makino *Enzymologia* 7 385 (1939)

Vitamin $B_6$ has also been obtained by a slightly more difficult route, starting with the ethoxy diamine (VII) By diazotization an ethoxy dihydroxy compound (XIV) is obtained, which upon hydrolysis with dilute hydrochloric acid gives vitamin $B_6$ When instead of the hydrochloric





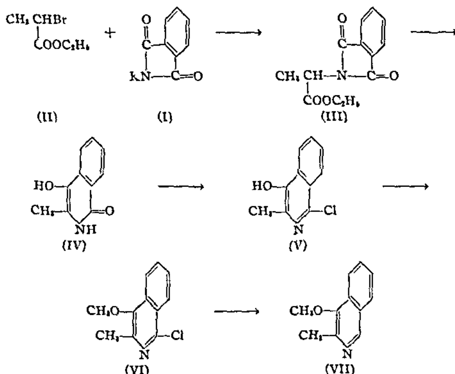

acid, hydrobromic acid is used, the dibromide (XV) is obtained, which requires another step to yield finally vitamin $B_6$

## (b) *Synthesis by Degradation of Isoquinoline*

The principle of this synthesis has independently been used by two groups of workers, by Kuhn and co workers[61] in Germany and by Itiba and Miti[62] in Japan, and consists of the oxidative degradation of 2 methyl 3 methoxy isoquinoline to 2 methyl 3 methoxy pyridine-4 5 dicarboxylic acid The isoquinoline derivative is prepared[63] by condensation of potas

[61] R Kuhn K Westphal G Wendt and O Westphal *Naturwissenschaften* 27 469 (1939)
[62] A Itiba and K Miti *Sci Papers Inst Phys Chem Research (Tokyo)* 36 173 (1939)
[63] S Gabriel and J Colman *Ber* 33 988 (1900)

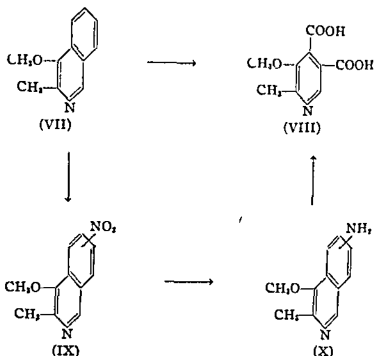
sium phthalimide (I) with α bromo propionic acid ester (II) to yield α phthalimide propionic acid ester (III) which upon saponification gives 2 methyl 3 hydroxy isocarbostyril (IV) Chlorination yields the chloro compound (V) which is converted into the *O* methyl ether (VI) by methylation with methyl iodide The latter compound upon treatment with tin and hydrochloric acid yields the 2 methyl 3 methoxy isoquinoline (VII)



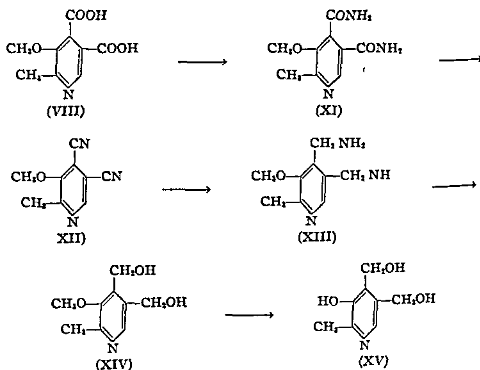

The 2 methyl 3 methoxy isoquinoline (VII) is converted into 2 methyl 3 methoxy pyridine 4 5 dicarboxylic acid (VIII) either by direct oxidation in alkali solution with permanganate[64] or by nitration to a Bz nitro compound (IX) followed by reduction to a Bz amino compound (X) which is then oxidized with permanganate to yield the dicarboxylic acid (VIII)[65]

The 2 methyl 3 methoxy pyridine-4 5 dicarboxylic acid (VIII) is converted through its diamide (XI) into 2 methyl 3 methoxy 4 5 dicyano pyridine (XII), which by catalytic hydrogenation yields the 2 methyl 3 methoxy 4 5 diamino methyl pyridine (XIII) The latter upon reaction with nitrite gives the vitamin $B_6$ *O* methyl ether (XIV) The methyl

[64] A Itiba and K M ti *Sci Papers Inst Phy Chem R search (Toky )* 36 173 (1939)
[65] R Kuhn K Westphal C Wendt and O Westphal *Naturwissenschaften* 27 469 (1939)




ether can be converted into the vitamin $B_6$ (XV) according to one of th previously mentioned methods



## 8 Industrial Methods of Preparation

Pure vitamin $B_6$ obtained by extraction of animal or plant material has never appeared on the market It has, however, been commercially available in the form of yeast or liver concentrates in mixture with several other members of the vitamin B complex In the future, extracts of this type will probably be prepared primarily as a source of the less well known members of the vitamin B group

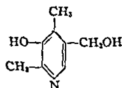
Vitamin $B_6$ is commercially available in the pure crystalline form, synthesized according to both methods outlined in the previous section dealing with the synthesis of vitamin $B_6$

## 9 Biogenesis

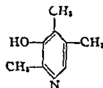
The biogenesis of vitamin $B_6$ is unknown Theories pertaining to the biogenesis of this vitamin have not been suggested

## 10 Specificity

Vitamin $B_6$ apparently owes its physiological action to the molecule as an entity Compounds of very similar structure and simple derivatives of vitamin $B_6$ are inactive The di and triacetate are fully active[66, 6] probably because the organism is able to hydrolyze these esters The benzoate however is inactive The same is true for the methyl ether,[67, 68] which however, shows some activity in a concentration that corresponds to about 500 times the concentration of the free vitamin[69] 4 Desoxy vitamin $B_6$ (2 4 dimethyl 3 hydroxy 5 hydroxy methyl pyridine (I)) appears to be active in 50 times the concentration of vitamin $B_6$[69, 70] and 4 5 bis desoxy vitamin $B_6$ (2,4 5 trimethyl 3 hydroxy pyridine (II)) appears to be inactive[69] A great number of other pyridine derivatives have been tested[69] but no active compound has been found



(I)
4 Desoxy vitamin $B_6$



(II)
4 5 Bis desoxy vitamin $B_6$

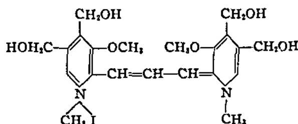
[66] R Kuhn and G Wendt *Ber* 71 1118 (1938)
[67] K Unna *Proc Soc Exptl Biol Med* 43 1 2 (1940)
[68] R Kuhn and G Wendt *Ber* 71 1534 (1938)
[69] L F Möller *Z phys l Chem* 260 246 (1939)
[70] F F Möller O Fim F Jung and T Moll *Naturwissenschaften* 27 2?8 (1939)

## 11 Determination

### (a) *Chemical Methods*

**Phenol Test According to Folin and Denis**[71, 72] This test consists in a blue color given by a phenolic hydroxyl group in the presence of a mixture of sodium tungstate, phosphotungstic acid phosphoric acid and lithium hydroxide By the use of this method 0 02–0 08 mg vitamin $B_6$ can be determined The color is measured in a photometer This method is not specific for vitamin $B_6$

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**Gibb's Phenol Indophenol Test** This test has been adapted to the quantitative colorimetric determination of vitamin $B_6$[73, 74] A blue color develops when a vitamin $B_6$ solution is mixed with a veronal buffer (pH 7 6) and a butanol solution of 2,6 dichloroquinone chloroimide The color has an absorption maximum at 660 mμ and is measured in a colorimeter or spectrophotometer

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[73] J V Scudi H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)
[74] J V Scudi K Unna and W Antopol *J Biol Chem* 135 371 (1940)

**Ferric Chloride Method**[75, 76] This method has proved to be of value in the estimation of vitamin $B_6$ in rich sources The red brown color developed by the unknown material is compared with those of standards

### (b) *Biological Methods*

**Yeast Growth Test** Since it has been observed that vitamin $B_6$ stimulates the growth of yeast, a method of determining this vitamin by the rate of growth of yeast has been proposed[77]

**Bacteria Test** *Streptobacterium plantarum* (*Bacterium Acetyl Cholini Keil* 10 S) responds in growth and production of acid to the amount of vitamin $B_6$ present The increase in growth is measured with a nephelometer[78, 79]

**Rat Growth Test** In this proposed test the growth rate increase of rats, following the administration of vitamin $B_6$, is determined[80, 81, 82, 83] Since the decline of the growth rate is not specific for a vitamin $B_6$ deficiency, this test often gives unsatisfactory results[85]

**Acrodynia Rat Test** Vitamin $B_6$ is assayed comparatively on the basis of the percentage incidence of the typical acrodynia type dermatitis in rats[84, 85, 86, 87, 88, 89, 90, 91, 92] in either prophylactic or curative assay procedures

## 12 Standard

One rat unit vitamin $B_6 = 10\ \gamma$, is defined as the amount necessary per day to cure or prevent the typical symptoms of avitaminosis (Gyorgy)[93]

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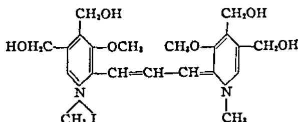
[75] J C Keresztesy and J P Stevens *J Am Chem Soc* 60 1267 (1938)
[76] R D Greene *J Biol Chem* 130 513 (1939)
[77] A S Schultz L Atkin and C N Frey *J Am Chem Soc* 61 1931 (1939)
[78] E F Möller *Z physiol Chem* 254 285 (1938)
[79] E F Möller O Fima F Jung and T Moll *Naturwissenschaften* 27 228 (1939)
[80] M K Dimick and C B Schreffler *J Nutrition* 17 23 (1939)
[81] R Kuhn and G Wendt *Z physiol Chem* 256 127 (1938)
[82] C E Edgar M M El Sadr and T F Macrae *Biochem J* 32 2207 (1938)
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P György and R E Eckardt *Biochem J* 34 1143 (1940)
[85] R C Bender and G C Supplee *J Am Chem Soc* 59 1178 (1937)
[86] P György *Biochem J* 29 760 (1935)
[87] G Lunde and H Kringstad *Ibid* 32 708 (1938)
[88] H I C Wilson and G K Roy *Indian J Med Research* 25 879 (1938)
[89] H A Schneider J K Ascham B R Platz and H Steenbock *J Nutrition* 18 99 (1939)
[90] N Halliday and H M Evans *J Biol Chem* 118 255 (1937)
M K Dimick and C B Schreffler *J Nutrition* 17 23 (1937)
[92] E J Reedman W L Sampson and K Unna *Proc Soc Exptl Biol Med* 43 112 (1940)
[93] P György *Ibid* 35 204 (1936)

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73 J V Scudi H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)
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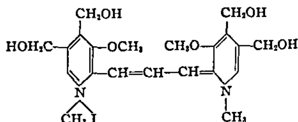
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[76] R D Greene *J Biol Chem* 130 513 (1939)
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[78] E F Möller *Z physiol Chem* 254 285 (1938)
[79] E F Möller O Fima F Jung and T Moll *Naturwissenschaften* 27 228 (1939)
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[74] J V Scudi K Unna and W Antopol *J Biol Chem* 135 371 (1940)

Little is known about the physiological action of vitamin $B_6$. It has been suggested that vitamin $B_6$ is connected with the utilization of unsaturated fatty acids.[107] It has also been observed that in vitamin $B_6$ avitaminotic rats, the livers are significantly heavier and contain a higher percentage of total fatty acids,[108] which increase cannot be correlated to the food intake.[109] Rats maintained on a vitamin $B_6$ deficient diet can according to some investigators be protected from the symptoms of vitamin $B_6$ deficiency by supplementing the diet with the essential unsaturated fatty acids.[110, 111] Others were unable to obtain this effect but called attention to the very similar type of dermatitis in both vitamin $B_6$ and essential fatty acid deficiency which, however, can be differentiated since edema occurs only in the case of vitamin $B_6$ deficiency. The crux of the problem of the relation of vitamin $B_6$ to the fat metabolism is that the animal organism (tested on rats) needs vitamin $B_6$ for the synthesis of fat from protein.[112] This vitamin is apparently concerned with the metabolism of the amino acids, as has also been shown in a determination of the protein and carbohydrate appetite in rats by the self selection method.[113]

The mechanism of the vitamin $B_6$ action is still unknown. Upon the discovery[114] that vitamin $B_6$ occurs in tissues partly bound to proteins it was suspected that the principal function of this vitamin is to act as part of some enzyme system, like some of the other members of the vitamin B group. The result of an approach to solve this problem was negative: the methyl iodo compound of vitamin $B_6$ is not reduced to a dihydro compound[115] in a manner similar to the reduction of the methyl iodo compound of nicotinamide (see page 238).

## 15 Avitaminosis and Hypovitaminosis

Vitamin $B_6$ avitaminosis in rats causes a specific symmetrical dermatitis which is called "acrodynia"[116] and which affects primarily the peripheral parts of the body such as the paws, the mouth, the tail, the ears and nose.[117]

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[107] T. W. Birch, *J. Biol. Chem.*, 124, 775 (1938).
[108] N. Halliday, *J. Nutrition*, 16, 285 (1938).
[109] R. W. Engel, *Proc. Am. Soc. Biol. Chem.*, 1941, XXXVII.
[110] F. W. Quackenbush and H. Steenbock, *Proc. XVI Intern. Physiol. Congr. Zurich*, 1938, 108.
[111] W. D. Salmon, *Proc. Am. Soc. Biol. Chem.*, 34, LXXXII (1940).
[112] E. W. McHenry and G. Gavin, *J. Biol. Chem.*, 138, 471 (1941).
[113] C. P. Richter and C. D. Hawkes, *Am. J. Physiol.*, 129, 459 (1940).
[114] R. Kuhn and G. Wendt, *Ber.*, 71, 780 (1938).
[115] R. Kuhn and I. Löw, *Ibid.*, 72, 1453 (1939).
[116] T. W. Birch, P. György and L. J. Harris, *Biochem. J.*, 29, 2830 (1935).
[117] W. W. Jefremow, *Voprosy Pitaniya*, 7, No. 3, 43 (1938). See *Chem. Zentr.*, I, 1939, 4496. *Voprosy Pitaniya*, 6, No. 1, 55 (1937). See *Chem. Zentr.*, I, 1938, 1152.

## 13 Physiology of Plants and Microorganisms

Although it can be assumed that vitamin $B_6$ is necessary *for plants and* microorganisms, only few actual facts are known It has been demon strated experimentally that vitamin $B_6$ is a nutrilite for yeast[94, 95] and *staphylococcus albus*[96] and that it is necessary for lactic acid bacteria[97] and for some hemolytic streptococci[98] Some other bacteria are able to synthesize vitamin $B_6$, for example, those in the rumen of sheep[99] Only a few observations concerning the vitamin $B_6$ action in higher plants are available Vitamin $B_6$ acts as a growth stimulant[100] on isolated tomato roots and cosmos[101] The isolated pea root does not require an external supply of vitamin $B_6$ and it must be presumed that the root is able to build up the vitamin from compounds present in the culture medium[101] Ana lytical determination of the vitamin $B_6$ content of wheat and maize plants seems to indicate a greater concentration of this vitamin in the germs and the integuments than in the endosperm[102]

## 14 Animal Physiology

Free vitamin $B_6$ is rapidly absorbed in the intestines[103] Vitamin $B_6$ bound to protein material is not absorbed Plant material, for example seeds and vegetables should be cooked to insure full utilization of the vitamin $B_6$ present[104] (as shown in experiments with rats)

*Investigations on the behavior of vitamin $B_6$ in the human organism* are scarce Vitamin $B_6$ has not been detected in blood[105] (a reinvestigation seems advisable) but is present in urine,[105, 106] which indicates that this com pound is of physiological importance for man The vitamin is also se creted in milk When excess amounts of vitamin $B_6$ are administered, they are largely destroyed in the organism Storage of this vitamin in the organism has not been observed

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94 A S Schultz L Atkin and C N Frey *J Am Chem Soc* 61 1931 (1939)
95 R E Eakin and R J Williams *Ibid* 61 1932 (1939)
96 S P Vilter and T D Spies *Science* 91 200 (1940)
97 E F Möller *Z physiol Chem* 254 285 (1938)
98 *B L Hutchings and D W Woolley Science* 90 41 (1939)
99 L W McElroy and H Goss *J Biol Chem* 130 437 (1939)
100 W J Robbins and M B Schmidt *Proc Natl Acad Sci U S* 25 1 (1939)
101 J Bonner *Am Chem Soc Div Agr Food Chem Meeting* Sept 1939 Abst p 13
102 *A M Copping Biochem J 30 849 (1936)*
103 J V Scudi K Unna and W Antopol *J Biol Chem* 135 371 (1940)
104 E M Lantz *New Mexico Agr Exptl Station Bull* No 268
105 Cited from W Stepp J Kühnau and H Schroeder *Die Vitamine* 2nd edition Stuttgart 1937 p 86
106 J V Scudi H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Ex ptl Biol Med* 43 118 (1940)

tion has also been called to the possibility that chilblains may afford a clinical demonstration of vitamin $B_6$ deficiency [139] Promising results have also been obtained in the treatment of myasthenia [137, 140] Improvement has furthermore been noted in patients with idiopathic epilepsy[137] and with macrocytic anemia of pellagra or pernicious anemia in relapse [141]

### (a) *Clinical Test Methods*

**The Urine Test** In urine the excretion of vitamin $B_6$ can be demonstrated for rats and for man [142] The methods used are either the chemical indophenol test or the biological rat test The former can be applied to urine when it contains 1 $\gamma$ per cc, but not when less material is present Urine also contains substances which interfere with the test In human beings vitamin $B_6$ can be demonstrated in the urine only after the intake of excessive doses The normal urinary excretion of man and dogs is less than 0 5 $\gamma$ per cc, while rats excrete about 0 5–1 0 $\gamma$ per cc

**The Blood Test** There is no method known by which vitamin $B_6$ can be determined in blood As a matter of fact the presence of this vitamin in blood has not been demonstrated as yet

## 16 Hypervitaminosis

Vitamin $B_6$ is a substance of relatively low toxicity [143, 144] Chronic toxicity was studied in rats, dogs and monkeys by daily feeding up to 10 mg per kilogram body weight over periods extending to three months No significant differences in weight or in the hemoglobin, erythrocytes, leucocytes etc, were observed Twenty mg per kilogram body weight, injected intravenously into cats, had no effect Single doses up to 1 g were tolerated without untoward effects Higher doses produced tonic convulsions and suggest involvement of certain parts of the nervous system The lethal dose in rats is about 3 g per kilogram body weight [140] Vitamin $B_6$ has a sedative effect in man [145]

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[139] P György *J Nutrition* 16 69 (1938)

[140] J V Scud H F Koones and J C Keresztesy *Proc Am Physiol Soc* 1940 163 *Proc Soc Exptl Biol Med* 43 118 (1940)

[141] R W Vilter H S Schiro and T D Spies *Nature* 145 388 (1940)

T D Spies R K Ladisch and W B Bean *J Am Med Assoc* 115 839 (1940)

[143] K Unna and W Antopol *Proc Soc Exptl Biol Med* 43 116 (1940)

[144] K Unna *Am J Physiol* 129 483 (1940)

[145] T D Spies D P Hightower and L H Hubbard *J Am Med Assoc* 115 292 (1940)

and which is accompanied by edema and scaliness [118] Furthermore, rats cease growing [119, 120] In dogs,[121] rats[122, 123, 124] and pigs[125, 126] fits of an epileptiform nature were observed besides the symptoms of subnormal growth and dermatitis The animals become abnormally excited and any extra stimulus induces fits The striated and cardiac muscles degenerate, and pathological changes have been noted in the nervous system, especially in the columns of the spinal cord Chicks also need vitamin $B_6$,[127, 128] but no characteristic dermatitis can be observed during times of vitamin $B_6$ deficiency The symptoms in chicks consist of slow growth, depressed appetite and inefficient utilization of food In some cases spastic convulsions and death were observed [129] In dogs, vitamin $B_6$ deficiency causes a microcytic hypochromic anemia[130, 131, 132, 133] and in rats a thymus atrophy has been observed [134] In rats kept on a vitamin $B_6$ deficient diet the accessory organs of reproduction are reduced and the animals show defective sexual behavior [135]

The present day knowledge of the action of vitamin $B_6$ in human beings is very limited The necessity of this vitamin for man has not been proved, but it seems reasonable to assume that vitamin $B_6$ is a vitamin in human nutrition Upon administration of vitamin $B_6$ to pellagrins, recovery has been observed in some cases in which the vitamins $B_1$, $B_2$ and nicotinic acid failed to remove the symptoms [136] These symptoms include nervousness insomnia, irritability, cramping pains in the stomach, muscular weakness and muscular rigidity [137] Patients with pseudohypertrophic muscular dystrophy, for example, respond well to vitamin $B_6$ treatment [138] Atten

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[118] W Antopol and K Unna *Proc Soc Exptl Biol Med* 42 126 (1939)
[119] R Kuhn and G Wendt *Z physiol Chem* 256 127 (1938)
[120] C E Edgar M M El Sadr and T F Macrae *Biochem J* 32 2207 (1938)
[121] P J Fouts O M Helmer S Lepkovsky and T H Jukes *J Nutrition* 16 197 (1938) J M McKibbin R J Madden S Black and C A Elvehjem *Am J Physiol* 128 102 (1939)
[122] H Chick A N Worden and M M El Sadr *J Soc Chem Ind* 58 1019 (1939)
[123] H Chick M M El Sadr and A N Worden *Biochem J* 34 595 (1940)
[124] J J Oleson H R Bird C A Elvehjem and E B Hart *J Biol Chem* 127 23 (1939)
[125] H Chick T F Macrae A J P Martin and C J Martin *Biochem J* 32 2207 (1938)
[126] M M Wintrobe *Am J Physiol* 126 375 (1939)
[127] D M Hegsted J J Oleson C A Elvehjem and E B Hart *J Biol Chem* 130 423 (1939)
[128] C W Carter and J R O Brien *Proc 7th World s Poultry Congress and Exposition* 1939 126
[129] T H Jukes *Proc Soc Exptl Biol Med* 42 180 (1939)
[130] P J Fouts O M Helmer S Lepkovsky and T H Jukes *J Nutrition* 16 197 (1938) P J Fouts O M Helmer and S Lepkovsky *Proc Soc Exptl Biol Med* 40 4 (1939)
[131] H J Borson and S R Mettier *Ibid* 43 429 (1940)
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[136] T D Spies W B Bean and W F Ashe *J Am Med Assoc* 112 2414 (1939)
[137] T D Spies D P Hightower and L H Hubbard *Ibid* 115 292 (1940)
[138] W Antopol and C E Schotland *Ibid* 114 1058 (1940)

# NICOTINIC ACID—
# NICOTINAMIDE

## 17 Requirements

Since vitamin $B_6$ has not yet been demonstrated as necessary for human life, no data are available as to the requirements of this vitamin for human beings In a few clinical cases daily doses of 10–100 mg were given [146]

The requirements for rats have been repeatedly investigated and were found to be 10 $\gamma$ daily[147, 148] of the vitamin hydrochloride Chicks also need this vitamin (about 30 $\gamma$ per day) [149] Sheep, however, do not require an external supply of vitamin $B_6$, since it is synthesized by bacteria in the rumen [150] The same has also been reported for cattle [151, 152]

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[146] T D Spies W B Bean and W F Ashe *J Am Med Assoc* 112 2414 (1939)

[147] R Kuhn and G Wendt *Ber* 71 780 (1938)

[148] M K Dimick and C B Schreffler *J Nutrition* 17 23 (1939)

[149] D M Hegsted J J Oleson C A Elvehjem and E B Hart *J Biol Chem* 130 423 (1939)

[150] L W McElroy and H Goss *Ibid* 130 437 (1939)

[151] L W McElroy and H Goss *Ibid* 133 LXV (1940)

[152] M I Wegner A N Booth C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 45 769 (1940)

# NICOTINIC ACID—
# NICOTINAMIDE

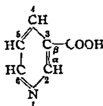
# NICOTINIC ACID—NICOTINAMIDE

## 1 Nomenclature and Survey

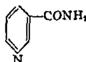
**Names**

Nicotinic acid and nicotinamide
Niacin  synonym for nicotinic acid
Niacin amide  synonym for nicotinamide
P P factor  Pellagra Preventive factor [1]
Pellagramine [2]
Niamid  Suggested abbreviation for nicotinamide

**Chemical formulas**



Nicotinic acid



Nicotinamide
(abbreviated formula)

**Chemical names**

Pyridine 3-carboxylic acid and acid amide
Pyridine β carboxylic acid and acid amide

**Empirical formulas**

Nicotinic acid  $C_6H_5O_2N$
Nicotinamide  $C_6H_6ON_2$

*Nicotinic acid may possibly be identical with vitamin $B_5$* The term vitamin $B_5$ was given originally to a fuller s earth eluate fraction from yeast and was shown to be necessary for the growth of pigeons Vitamin $B_6$ was later found to be present in vitamin $B_5$ fractions but could not fully replace the vitamin $B_5$ requirements of pigeons The remaining factor designated as vitamin $B_5$ appears to be identical with nicotinic acid [3]

[1] The term pellagra preventive factor was appl ed to the substance originally po tulated a necessary for the prevention of human pellagra J Goldberger and W F Tanner *U S Pub Health Service Pub Health Repts* 39 87 (19 4)

[2] R L Jones *Science* 68 480 (19 8)

[3] C. W Carter and J R O Brien *Bioch m J* 33 1810 (19 9)

## 2 Chronology

1735 First description of the human disease pellagra by CASAL

1907 SEARCY reported the occurrence of pellagra in epidemic forms

1912–1914 FUNK[4] in Europe and SUZUKI[5] in Japan isolated nicotinic acid from yeast and rice bran during their search for the antipolyneuritic vitamin (vitamin $B_1$) but failed to recognize the vitamin character of the isolated compound FUNK observed however the beneficial effect of nicotinic acid when given in mixture with the antipolyneuritic vitamin

1917 CHITTENDEN and UNDERHILL[6] produced a pathological condition resembling human pellagra in dogs which is called canine blacktongue

1926 GOLDBERGER and LILLIE obtained an experimental pellagra like condition in albino rats by feeding a diet deficient in a substance called Pellagra Preventive Factor (P P Factor) [7]

1928 GOLDBERGER and associates[8] collected evidence that substances capable of curing blacktongue in dogs were equally effective in the cure of human pellagra

1929 AYKROYD and ROSCOE recognized that the pellagra preventive factor and the anti blacktongue factor always occur together [9]

1930 NORRIS and RINGROSE[10] produced a pellagra like condition in chicks

1935 WARBURG and CHRISTIAN[11] and EULER ALBERS and SCHLENK[12] demonstrated that nicotinamide is a part of the hydrogen transporting coenzymes

1936 EULER and MALMBERG[13] reported that nicotinic acid prolonged the lives of rats fed vitamin $B_1$ vitamin $B_2$ and vitamin $B_4$

1937 KNIGHT[14] and MUELLER[15] found nicotinamide to be essential to the growth of certain unicellular organisms ELVEHJEM and co-workers[16] showed that nicotinic acid is effective in the cure of blacktongue in dogs and that it can be isolated from anti blacktongue active liver extracts FOUTS HELMER LEPKOVSKY and JUKES[17] reported the first successful treatment of human pellagra with nicotinamide

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[4] C Funk *J Physiol* 46 173 (1913) *Brit Med J* 1913 I 814 J C Drummond and C. Funk *Biochem J* 8 594 (1914)

[5] U Suzuki and S Matsunaga *J Agr Tokyo Imp Univ* 5 59 (1912) U Suzuki T Shamimura and S Okade *Biochem Z* 43 89 99 (1912)

[6] R H Chittenden and F P Underhill *Am J Physiol* 44 13 (1917)

[7] J Goldberger and R D Lillie *U S Pub Health Service Pub Health Repts* 41 10 5 (1926)

[8] J Goldberger and G A Wheeler *Ibid* 43 172 (1928) J Goldberger G A Wheeler R. D Lillie and L M Rogers *Ibid* 43 1385 (1928) J Goldberger G A Wheeler L M Rogers and W H Sebrell *Ibid* 45 1297 (1930)

[9] W R Aykroyd and M H Roscoe *Biochem J* 23 483 (1929)

[10] L C Norris and A T Ringrose *Science* 71 643 (1930)

[11] O Warburg and W Christian *Biochem Z* 275 464 (1935) O Warburg W Christian and A Griese *Ibid* 279 143 (1935)

[12] H v Euler H Albers and F Schlenk *Z physiol Chem* 237 1 (1935)

[13] H v Euler and M Malmberg *Biochem Z* 284 455 (1936)

[14] B C J C Knight *Biochem J* 31 731 (1937)

[15] J H Mueller *J Bact* 34 429 (1937) *J Biol Chem* 120 219 (1937)

[16] C A Elvehjem R J Madden F M Strong and D W Woolley *J Am Chem Soc* 59 1767 (1937) *J Biol Chem* 123 137 (1938)

[17] P J Fouts O M Helmer S Lepkovsky and T H Jukes *Proc Soc Exptl Biol Med* 37 405 (1937)

## 3 Occurrence of Nicotinic Acid and of Nicotinamide

Nicotinic acid occurs in all living cells in small amounts The liver [18] the adrenal gland[19] and yeast and wheat germs are especially rich in nicotinic acid The eye lenses contain a fair amount[19] and corn meal corn syrup alfalfa [20] fatty meat and milk [21] contain small amounts

Free nicotinic acid apparently does not occur in the living organism but is found in the urine of animals Nicotinic acid occurs in tissues in the form of its amide Besides the occurrence of the free nicotinamide there exists a number of enzyme systems (see page 227) in which nicotinamide is chemically bound Nicotinamide occurs to a much greater extent in the bound form than as the free nicotinamide Thus in rats bound nicotinamide occurs for example in the liver, kidney and muscles while free nicotinic acid (or its amide) has been found only in the liver

## 4 Isolation of Nicotinic Acid and of Nicotinamide

**The isolation of nicotinic acid** from natural sources is a relatively simple matter Tissue material is freed from fats by extraction with organic solvents The remaining material is saponified, preferably with alkali The acid fraction of the saponification mass contains the nicotinic acid which can be separated as such as the ester or as the Cu salt From this salt the free acid is obtained by means of hydrogen sulfide

**The isolation of the total nicotinamide** from animals or plants is carried out by water extraction of the material followed by partial hydrolysis in 0 1 *N* sulfuric acid in order to split the nicotinamide from its chemical combination with the various enzymes The water phase is then extracted with butanol or chloroform [2] The chloroform solution is subjected to fractional distillation Nicotinamide distills at 150–160° C at 5 × $10^{-4}$ mm Hg [3] The distillate may be recrystallized from chloroform and benzene

**The separation of free nicotinic acid from nicotinamide** is effected by solvent extraction (ether chloroform butanol) of a water solution The amide is soluble in organic solvents while the free acid remains in the water phase

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[18] W J Dann *Science* 86 616 (1937)
[19] E Kodicek *J Soc Chem Ind* 58 1088 (1939)
[20] W R Wyatt *Iowa State Coll J Sci* 14 103 (1939)
[21] E Kodicek *Biochem J* 34 712 724 (1940)
[2] H v Euler F Schlenk L Melzer and B Högberg *Z physiol Chem* 258 21 (1939) P Karrer and H Keller *Helv Chim Acta* 22 1292 (1939)
[3] R Kuhn and H Vetter *Ber* 68 2374 (1935)

The separation of free nicotinamide from chemically bound nicotinamide (coenzymes) must be carried out immediately after death,[24,25] and is usually achieved by an acetone extraction of the material Acetone dissolves nicotinamide, but does not dissolve the coenzymes Acetone, furthermore, prevents the naturally occurring enzyme systems from splitting the nicotinamide containing coenzymes into their components

## 5 Properties of Nicotinic Acid and of Nicotinamide

1 Nicotinic acid crystallizes in needles from water or alcohol and melts at 235 5–236 6° C [6] It sublimes without decomposition Nicotinic acid exhibits a typical absorption spectrum with a maximum at 385 mμ [27]

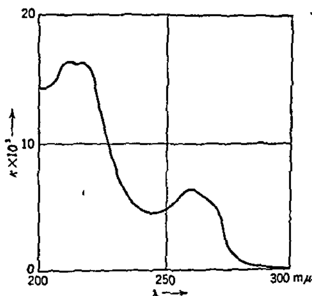



Fig 12 —Absorption spectrum of nicotinamide in water (R Kuhn and H Vetter)

2 Nicotinamide crystallizes in needles from benzene and melts at 129° C It distills at 150–160° C and $5 \times 10^{-4}$ mm Hg [28] The absorption spectrum of nicotinamide is shown in Fig 12

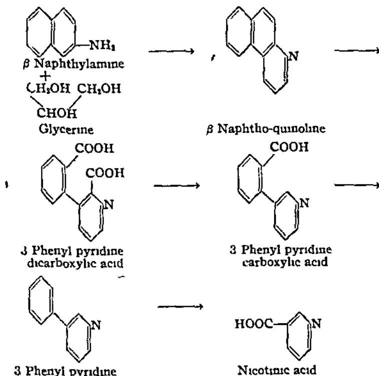
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[24] H v Euler F Schlenk L Melzer and B Högberg *Z physiol Chem* 258 212 (1939)
[25] H v Euler and K Myrbäck *Ibid* 117 237 (1928) H v Euler and G Günther *Ibid* 243 1 (1936) H v Euler H Heiwinkel and F Schlenk *Ibid* 247 IV (1937)
[26] R. Gording and L A Flexser *J Am Pharm Assoc* 29 230 (1940)
[27] H Hünecke *Ber* 60 1451 (1927)
[28] R. Kuhn and *H Vetter Ibid* 68 2374 (1935)

### 6 Constitution of Nicotinic Acid and of Nicotinamide

The constitution of nicotinic acid was determined when this acid was first obtained by oxidation of nicotine [9] from which the name of this acid originates

The acid character was established by the formation of a silver and a copper salt and by the formation of various derivatives such as esters acid chloride etc The basic character was recognized by the formation of crystallized salts such as the hydrochloride the hydrobromide etc By distillation of the calcium salt of the acid the carboxylic acid group is split off and pyridine is obtained [10]




The *m* or 3 position of the carboxylic acid group in reference to the ring nitrogen was suspected by Skraup who investigated the physical constants and the decarboxylation of the three possible pyridine mono carboxylic acids picolinic acid (*o* or 1,2 ) nicotinic acid (*m* or 1 3 ) and γ pyridine carboxylic acid (*p* or 1 4 position) [11] The *m* position was proved to be the correct one upon oxidation of the synthetically prepared 3 phenyl

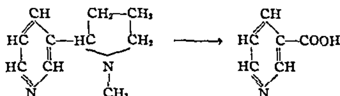
[9] C. Huber *Be* 3 849 (1870) *Ann* 141 71 (1867) H Weidel *Ann* 165 346 (1873)
[10] H Weidel *Ann* 165 331 (1873)
[11] Z H Skraup *Monatsh* 1 800 (1880)

pyridine the constitution of which is beyond doubt 3 Phenyl pyridine was prepared from β naphtho quinoline which in turn was synthesized from β naphthylamine and glycerine β Naphtho quinoline yields upon *oxidation with permanganate* β phenyl pyridine dicarboxylic acid, which by stepwise decarboxylation yields the mono carboxylic acid and finally 3 phenyl pyridine [32] A further proof for the 3 position of the carboxylic acid group in nicotinic acid is its formation from the synthetically prepared *m* dipyridyl [33]

## 7 Synthesis

### (a) *Nicotinic Acid*

1 **By Oxidation of Nicotine** This is the method by which nicotinic acid was discovered The oxidation may be accomplished by fuming nitric acid,[34] by chromic acid[35] or by permanganate [36]



2 **By Oxidation of β-Pyridines** This method is really a generalization of the method first discussed β Picoline,[37] 3 ethyl pyridine,[38] 3 phenyl pyridine,[39] 3,3′ dipyridyl[40] and similar compounds have been converted by this method into nicotinic acid

3 **By Decomposition of Pyridine-poly-carboxylic Acids** Any pyridine poly carboxylic acid, which has one carboxylic acid group in 3 position can be converted into the 3 mono carboxylic acid by thermal decomposition[41] or by acidic[42] decomposition An exception to this rule

[32] Z H Skraup and A Cobenzl *Monatsh* 4 436 (1883)

[33] Z H Skraup and G Vortmann *Ibid* 4 594 (1883)

[34] H Weidel *Ann* 165 331 (1873)

[35] C Huber *Ber* 3 849 (1870) *Ann* 141 271 (1867) H Weidel *Ann* 165 346 (1873)

[36] R Laiblin *Ber* 10 2136 (1877) *Ann* 196 135 (1879)

[37] H Weidel *Ber* 12 1992 2004 (1879) H Ost *J prakt Chem* [2] 27 286 (1883) F Seyfferth *Ibid* [2] 34 258 (1886)

[38] H Weidel and K Hazura *Monatsh* 3 783 (1882) A Ladenburg *Ann* 301 152 (1898) A Wischnegradski *Ber* 12 1480 (1879)

[39] Z H Skraup and A Cobenzl *Monatsh* 4 458 (1883)

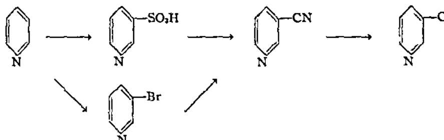
[40] Z H Skraup and G Vortmann *Ibid* 4 594 (1883)

[41] S Hoogewerff and W A van Dorp *Ann* 204 117 (1880) *Ibid* 207 219 226 (1881) *Rec trav chim* 1 122 (1882) R Camps *Arch Pharm* 240 353 359 (1902) H Weidel and J Herzig *Monatsh* 1 16 (1880) F B Ahrens and R Gorkow *Ber* 37 2063 (1904)

[42] H Ost *J prakt Chem* [2] 27 286 (1883) S Hoogewerff and W A van Dorp *Ber* 14 974 (1881) H Weidel and J Herzig *Monatsh* 6 987 (1885)

is the 3 4 dicarboxylic acid which decarboxylates only under very conditions yielding pyridine 4 carboxylic acid

4 **By Synthesis from Pyridine** This is a total synthesis of n acid Pyridine is sulfonated with fuming sulfuric acid, yielding sulfonic acid By distillation of its sodium salt with potassium c nicotinic acid nitrile is obtained [43]




Considerably better yields are obtained by first brominating pyri the 3 position followed by conversion into 3 cyano pyridine by m cuprous cyanide [44] Saponification of the nicotinonitrile yields n acid

*(b) Nicotinamide*

1 **By Amidation of Nicotinic Acid** Nicotinamide is obtai passing ammonia gas into nicotinic acid at 230° C [45]

2 **By Amidation of Esters of Nicotinic Acid** The methyl or ester of nicotinic acid yields nicotinamide on reaction with aque better, alcoholic ammonia [46]

## 8 Industrial Methods of Preparing Nicotinic Acid and Nicoti

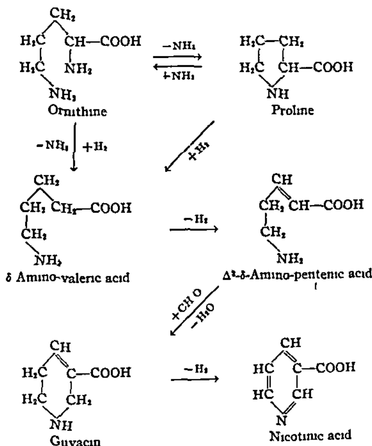
Nicotinic acid and its amide are prepared according to the n described before The nitric acid oxidation of nicotine, a relatively alkaloid obtained as a by product from tobacco, is of special in Alternative procedures are permanganate oxidations of $\beta$ pico quinoline For technical purposes the oxidation of the fraction o from coal tar or petroleum which distills between 135° and 142° C [4 oxidation of the corresponding fraction of bone tar oil[49] has been cons

[42] O Fischer *Ber* 15 63 (188 )
[43] S M McElvain and M A Goese *J Am Chem Soc* 63 283 (1941)
[44] S Ke matsu K. Yok ta and I Satoda *J Pha m Soc Japan* 53 994 (1933)
[45] C Engler *Ber* 27 1787 (1894) R Camps *Arch Pharm* 240 354 (190?) F Po *natsh* 16 53 (1895) F B La Forge *J Am Chem Soc* 50 2477 (19?8)
[46] *Org Syntheses* Coll Vol 1 378 (1932)
[47] A. Pinner *Ber* 33 1227 (1900)
[48] H Weidel *Ibid* 12 1992 (1879)

A number of *N* substituted nicotinamide derivatives have attracted commercial interest because of their clinical use in the treatment of shock, collapse and cardiac decompensation [50] The most useful compound of these derivatives is the *N* diethyl nicotinamide

## 9 *Biogenesis of Nicotinic Acid*

The biogenesis of nicotinic acid is not known with certainty, but is believed to be linked with the amino acid metabolism It has been suggested[51] that nicotinic acid may originate from ornithine or proline The first reaction product would be δ amino valeric acid This reaction is common for bacteria[52] but has not been proved for plants The further course of the suggested nicotinic acid synthesis is indicated in the following scheme




[50] A selected number of patents for the preparation and use of these compounds appear in the patent index at the end of the book

[51] M Guggenheim *Die biogenen Amine* New York 1940 p 174

[52] D Ackermann *Z Biol* 57 104 (1910) C Neuberg *Biochem Z* 37 490 (1911) E Salkowski and H Salkowski *Ber* 16 1191 (1883)

## 10 Enzyme Systems Containing Nicotinamide

All plant and animal cells contain among their enzyme systems c dehydrogenases which transport hydrogen and take care of a numl different dehydrogenation reactions The classical conception is each of these enzymes or, better, holoenzymes consists of an apoer and a coenzyme The apoenzyme, which is believed to have no cat properties of its own, is the protein bearer of the coenzyme, which garded as the prosthetic group of the protein [53] Another view is th protein is the enzyme itself and that the coenzyme merely acts as a sp substrate to accept hydrogen [54] There are two coenzymes known o class of dehydrogenases, namely codehydrogenases I and II, whic also called coenzymes I and II The number of apoenzymes combine with these two coenzymes is considerably greater It is bel that the two coenzymes need different apoenzymes for their acti

TABLE I

| Substrate system | Apoenzyme source | Coe |
|---|---|---|
| β Hydroxy butyrate ⇌ acetoacetate[55] | Cardiac muscle | I |
| Formate → $CO_2 + H_2O$[56] | Dried pea seeds | I |
| Lactate ⇌ pyruvate[57] | Skeletal muscle | I |
| Malate ⇌ oxaloacetate[57] | Skeletal muscle | I |
| Alcohol ⇌ acetaldehyde | Yeast[58, 59, 60] | I |
| | Liver[61] | I |
| Glucose → gluconic acid | Liver[62] | I o |
| Glutamic acid ⇌ α keto glutaric acid + $NH_3$[54, 64] | Plants | I |
| | Yeast | I |
| | Liver | I o |
| 2 Aldehydes → 1 alcohol + 1 acid[7] (aldehyde mutation) | Liver | I |
| α Glycerophosphate → phosphoglycerate[68] | Skeletal intestinal and cardiac muscle | I |
| Phospho glyceraldehyde ⇌ diphospho glycerate[69] (triose catabolism)[70] | Skeletal and cardiac muscle brain | I |
| Glucose 6 phosphate → 6 phospho gluconate[1] | Yeast erythrocytes | I |
| 6 Phospho-gluconate → phospho keto hexonate[72] | Yeast animal tissues | I |
| Citrate → α keto-glutarate[73] | Liver heart | I |

*(See following page for table footnotes)*

[53] O Warburg *Ergeb Enzymforsch* 7 210 (1937)
[54] M Dixon and L G Zerfas *Biochem J* 34 371 (1940)

oxidizing and as reducing agents Specific proteins are used for each substrate and a specific protein may in special cases dehydrogenate the same substrate with different coenzymes

Table I summarizes the better known dehydrogenation reactions in which the codehydrogenases participate It is evident that these co enzymes are involved in a wide variety of reactions There are quite probably other such reactions, which have not been thoroughly investi gated It has, for example, been postulated that the oxidation of cysteine to cystine involves the participation of a codehydrogenase [74]

During the course of the dehydrogenation reactions indicated in the table, the coenzymes are reduced to dihydro compounds The reverse reaction the oxidation of the dihydro codehydrogenases to the code hydrogenases, is carried out in the presence of different apoenzymes as previously stated Principally all dehydrogenation reactions are re versible, although in living tissues usually no such equilibrium occurs due to the fact that the reaction products do not accumulate but undergo further reaction Practically, the equilibrium can be demonstrated in many cases such as in the system involving alcohol and acetaldehyde and is indicated in the table above for those systems for which the reversible reaction has been experimentally demonstrated Equilibrium constants have been determined for the coenzyme I and for many of the reactions

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[55] D E Green J G Dewan and L F Leloir *Biochem J* 31 934 (1937) D E Green and J G Dewan *Ibid* 31 1069 1074 (1937)

[56] E Adler and M Sreenivasaya *Z physiol Chem* 249 24 (1937)

[57] D E Green D M Needham and J C Dewan *Biochem J* 31 2327 (1937) D E Green and J Brosteaux *Ibid* 30 1489 (1936) D E Green *Ibid* 30 2095 (1936)

[58] E Negelein and H J Wulff *Biochem Z* 289 436 (1937) 293 351 (1937)

[59] O Warburg and W Christian *Helv Chim Acta* 19 E 79 (1936)

[60] H v Euler E Adler and H Hellström *Z physiol Chem* 241 239 (1936)

[61] C Lutwak Mann *Biochem J* 32 1364 (1938)

[62] D C Harrison *Ibid* 25 1016 (1931) F Adler and H v Euler *Z physiol Chem* 232 6 (1935)

[63] N B Das *Z physiol Chem* 238 269 (1936)

[64] H v Euler F Adler G Günther and N B Das *Ibid* 259 61 (1938)

[65] E Adler N B Das and E v Euler *Sr Vet Akad Ark Kemi* 12 1 (1937) J G Dewan *Biochem J* 32 1378 (1938) H A Krebs and P P Cohen *Ibid* 33 1895 (1939)

[66] H v Euler E Adler and T S Eriksen *Z physiol Chem* 248 227 (1937) F Adler G Günther and J E Everett *Ibid* 255 27 (1938)

[67] M Dixon and C Lutwak Mann *Biochem J* 31 1347 (1937) M Dixon *Ergeb Enzymforsch* 8 217 (1939)

[68] H v Euler E Adler and G Gunther *Z physiol Chem* 249 1 (1937)

[69] O Warburg and W Christian *Biochem Z* 301 221 (1939) 303 40 (1939)

[70] D E Green D M Needham and J D Dewan *Biochem J* 31 2327 (1937)

[71] O Warburg and W Christian *Biochem Z* 242 206 (1931) 254 438 (1932) F Negelein and W Gerischer *Ibid* 284 289 (1936)

[72] F Dickens *Biochem J* 32 1626 (1938) F Lipmann *Nature* 138 588 (1936) O Warburg and W Christian *Biochem Z* 287 440 (1936) 292 287 (1936)

[73] E Adler H v Euler G Günther and M Plass *Biochem J* 33 1028 (1939)

[74] E Maschmann *Naturwissenschaften* 27 628 (1939)

catalyzed by the coenzymes and are usually expressed in the form of the oxidation reduction potential[75] The potential $E_0'$ for the coenzyme I at 30° C is approximately −0 29 volts[76]

In tissues the oxidation of the dihydro coenzymes may be accomplished by coenzyme linked reactions[77] It is for example possible that β hydroxy butyrate is oxidized to acetoacetate (see Table I) with the formation of dihydro cozymase I which in turn reduces an aldehyde to an alcohol Thus the following compounds have been shown to act as acceptors in the presence of the corresponding apoenzymes acetaldehyde, pyruvate, oxaloacetate, triose phosphate imino glutarate (or α keto glutarate + $NH_3$) and fumarate[77] (in the presence of succinic dehydrogenase) It has also been shown that the riboflavin containing enzyme systems (see page 171) may be linked with the oxidation of the dihydro codehydrogenases

## 11 Coenzymes Containing Nicotinamide

### (a) *Codehydrogenase I*

**Synonyms** Codehydrogenase I Coenzyme I, Cozymase Coferment I, Diphosphopyridine nucleotide Coferment of fermentation, Coreductase Factor V[78, 79, 80]

**Occurrence** Codehydrogenase I has been found in all animal and plant cells in which carbohydrates are metabolized Yeast and red blood cells are especially rich sources and some muscles for example, heart muscles contain relatively high amounts In fresh yeast about 0 5 g of codehydrogenase I is present per kilogram[81] and in the heart muscle of rabbits 0 4 g per kilogram[8] The same amount (0 1–0 4 g) has been calculated to be present in muscles of man[83] and of invertebrata[84] Codehydrogenase I occurs also in microorganisms and has for example, been obtained from *Azotobacter chroococcum*[85] There seems to be a fairly con

[75] W M Clark *The Determination of Hydrogen Ion Concentration* Baltimore 1928 I Michaelis *Oxidation Reduction Potentiale* Berlin 1933

[76] F Schlenk H Hellström and H v Euler *Ber* 71 1471 (1938) H Borsook *J Biol Chem* 133 629 (1940)

[77] J G Dewan and D E Green *Biochem J* 31 1074 (1937)

[78] A Lwoff and M Lwoff *Proc Roy Soc (Lond n)* B122 359 360 (1937) *Compt rend* 203 520 (1936)

[79] H I Kohn *Biochem J* 32 2075 (1938)

[80] T M Rivers *Bull Johns Hopkins Hosp* 33 149 429 (1922)

[81] O Meyerhof and P Ohlmeyer *Biochem Z* 290 334 (1937)

[82] H v Euler *Angew Chem* 50 831 (1937)

[83] H v Euler *Atti 10th Cong Intern Chem* 1 178 (1938)

[84] S Ochoa and C G Ochoa *Nature* 140 1097 (1937)

[85] R Nilsson *Arch Mikrobiol* 7 598 (1937)

stant ratio of coenzyme to dihydro coenzyme in the muscles of all animals, the reduced form being present in about 35–45% of the total amount [84] An increased amount of the reduced form has been found in Jensen sarcoma [86, 87]

**Isolation** [88] The isolation of codehydrogenase I is carried out by water extraction of the source, for example, of yeast or red blood cells It is necessary to destroy some of the other enzymes present prior to the extraction, by short heating to about 80° C, since otherwise the code hydrogenase is rapidly destroyed After filtration or dialysis some of the protein impurities are removed by precipitation with lead acetate The coenzyme itself may be extracted with phenol[89] and is precipitated by mercuric acetate or nitrate picric acid, phosphotungstic or silicotungstic acid (and decomposed by ether amyl alcohol sulfuric acid), by silver salts either in ammoniacal solution or in a solution containing barium hydroxide (and freed from silver by hydrogen sulfide), by cuprous halides in the presence of hydrochloric acid (and freed from copper by hydrogen sulfide), by ethyl acetate *from an acidified methanol solution*, by acetone, and by alcohol The latter is also used for fractional precipitation of this coenzyme Purification may also be accomplished by fractional adsorption on aluminum oxide or charcoal from weakly acid solutions

The methods used for the separation of nicotinamide from the coenzymes have been described in the section on the isolation of nicotinamide (page 222) The methods used for the separation of codehydrogenase I from codehydrogenase II will be found in the section on the isolation of code hydrogenase II (page 235) To separate the flavin adenine dinucleotides (see page 179) which occur together with the codehydrogenases I and II in the phenol extract from certain sources such as yeast, the flavin compounds are precipitated in acid solution as silver salts [89, 90]

**Properties** Codehydrogenase I is a colorless, water soluble substance, and is insoluble in most organic solvents It exhibits a characteristic absorption spectrum with a maximum at 260 mμ [91] Hydrogenation to the dihydro codehydrogenase, which occurs during the enzyme action (see page 238), changes the absorption spectrum characteristically with the

[86] H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938)

[87] H v Euler M Malmberg and G Günther *Z Krebsforsch* 45 425 (1937)

[88] H v Euler *Ergeb Physiol* 38 1 (1936) O Warburg and W Christian *Biochem Z* 287 291 (1936) O Meyerhof *Ergeb Physiol* 39 10 (1937) H v Euler H Albers and F Schlenk *Z physiol Chem* 260 113 (1936) *Fortschr Chem org Naturstoffe* 1 99 (1938) P Ohlmeyer *Biochem Z* 297 66 (1938) H v Euler and E Adler *Z physiol Chem* 238, 233 (1936) B J Jandorf *J Biol Chem* 138 305 (1941) K Myrbäck and H Larsson *Z physiol Chem* 225 131 (1934)

[89] O Warburg and W Christian *Biochem Z* 298 150 *377 (1938)*

[90] J R Klein *J Biol Chem* 134 43 (1940)

[91] O Warburg and W Christian *Helv Chim Acta* 19 E 79 (1936) H v Euler E Adler and H Hellström *Z physiol Chem* 241 239 (1936) K Myrbäck H v Euler and H Hellström *Ibid* 212 7 (1932)

appearance of an additional band at 320–360 mμ with a maximum at 340 mμ (see Fig 13)

While the codehydrogenase I shows no fluorescence the dihydro compound exhibits a strong whitish fluorescence upon irradiation with ultra violet light

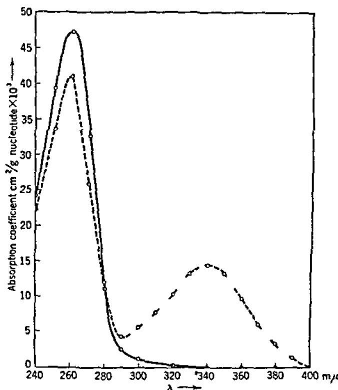



Fig 13—Absorption spectrum of codehydrogenase II in oxidized (——) and in reduced (- - - -) form (O Warburg and W Christian)

Codehydrogenase I is optically active the specific rotation being approximately −20° for the red cadmium line (643 9 mμ)[9] and −70° for the green mercury line (546 mμ)

Codehydrogenase I is quite stable in acid solution at moderate temperatures[93] whereas the dihydro codehydrogenase is destroyed by acids In alkaline solution codehydrogenase is rapidly destroyed[94] whereas the dihydro codehydrogenase remains unchanged when heated for 30 minutes

[9] K Myrbäck H v Euler and H Hellström *Z physiol Chem* 212 7 (1932)
K Myrbäck *Ibid* 234 259 (1935)
[9] K Myrbäck and B Örtenblad *Ibid* 234 254 (1935)

of codehydrogenase is the determination of the total hydrogen consumption upon catalytic hydrogenation in the presence of sodium borate

**Determination by Biochemical Methods** Codehydrogenase I and its dihydro form are usually determined by the degree of activation which they exert on fermentation[114, 115, 116] in the presence of an excess of the apodehydrogenase The criterion is the amount of $CO_2$ evolved under specified conditions which is proportional to the codehydrogenase I concentration This method is somewhat unreliable due to the fact that the purity of the apoenzyme is not standardized[117] This method is specific for codehydrogenase I and codehydrogenase II Nicotinic acid and its amide do not respond to this test

Another biochemical assay procedure for codehydrogenase I is based upon the fact that the oxidation of lactic acid by animal tissues requires the presence of codehydrogenase I[118]

**Determination by Biological Methods** *Bacillus influenzae* can be used to measure accurately the total content of codehydrogenases in blood and of yeast (and probably of other sources) since this bacillus cannot synthesize the codehydrogenases from their constituents but needs the coenzymes for proper development[119, 120, 121]

**Standard** One unit of codehydrogenase I is defined as that quantity which produces 1 cc of carbon dioxide in a normal fermentation[12] under specified conditions

### (*b*) *Codehydrogenase II*

**Synonyms** Triphosphopyridine nucleotide Warburg s Coferment, Respiratory coenzyme, Growth factor V[123, 124, 125]

**Occurrence** Codehydrogenase II seems, like codehydrogenase I, to occur in practically all living cells These apparently have the power

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[114] K Myrbäck *Z physiol Chem* 177 158 (1928)

[115] A E Axelrod and C. A Elvehjem *J Biol Chem* 131 77 (1939)

[116] B J Jandorf F W Klemperer and A B Hastings *Ibid* 138 311 (1941)

[117] H v Euler and K Myrbäck *Z physiol Chem* 190 93 (1930)

[118] D E Green and J Brosteaux *Biochem J* 30 1489 (1936)

[119] A Lwoff and M Lwoff *Proc Roy Soc (London)* B122 352 360 (1937) *Compt rend* 203 520 (1936)

[120] R W Vilter S P Vilter and T D Spies *J Am Med Assoc* 112 420 (1939)

[121] *H I Kohn Biochem J 32 2075* (1938)

[122] H v Euler H Albers and F Schlenk *Z physiol Chem* 240 113 (1936) H v Euler and K Myrbäck *Ibid* 136 108 (1924) H v Euler and S Karlson *Ibid* 123 93 (192?)

[123] T M Rivers *Bull Johns Hopkins Hosp* 33 149 429 (1922)

[124] H I Kohn *Biochem J* 32 2075 (1938)

[125] A Lwoff and M Lwoff *Proc Roy Soc (London)* B122 352 360 (1937) *Compt rend 203 520* (1936)

of synthesizing both codehydrogenases (see under Synthesis, page 233) from nicotinic acid It has also been postulated that the living cell is able to convert codehydrogenase I into codehydrogenase II It seems plausible, therefore that both coenzymes are found together It is noteworthy that the ratio of the amounts of the two coenzymes may vary considerably in different sources While yeast contains very little code hydrogenase II, animal tissue contains as much as 40–80 $\gamma$ per gram [126]

**Isolation** The isolation of codehydrogenase II is carried out for example, from washed red blood cells, by destruction of the cell structure followed by a combination of various precipitation reactions [127, 128] Code hydrogenase II is precipitated from a water solution by acetone, by ethyl acetate especially from a methanol HCl solution by mercuric acetate by barium salts by lead salts etc

**Separation of Codehydrogenase I from Codehydrogenase II** The following methods have been recommended for the separation of the two coenzymes

1 Codehydrogenase I is separated as the cuprous salt[179] whereby code hydrogenase II remains in solution and can be isolated separately

2 Codehydrogenase II is precipitated by lead acetate codehydro genase I is not [130]

3 Codehydrogenase II is more strongly adsorbed on aluminum oxide than codehydrogenase I [129] The codehydrogenase II is eluted from the $Al_2O_3$ by $KH_2PO_4$ solutions

4 The barium salts of the two codehydrogenases can be separated by fractional precipitation with alcohol [131]

**Properties** The properties of codehydrogenase II resemble very closely those of codehydrogenase I They are colorless water soluble compounds which are insoluble in organic solvents Codehydrogenase II is soluble in organic solvents in the presence of hydrochloric acid for example, in methanol HCl

Codehydrogenase II exhibits the same characteristic absorption band at 260 mμ as does codehydrogenase I The maximum of the typical dihydro codehydrogenase absorption is at 340 mμ[131] (see Fig 13) The latter compound also shows the characteristic fluorescence when irradiated

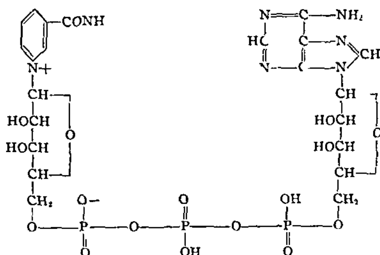
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[126] H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938)
[127] F Schlenk *Tab Biol* 14 186 (1937)
[128] O Warburg W Christian and A Griese *Biochem Z* 282 157 (1935)
[129] H v Euler and E Adler *Z physiol Chem* 238 233 (1936)
[130] H v Euler *Ibid* 240 113 (1936)
[131] O Warburg and W Christian *Biochem Z* 287 291 (1936)

with ultraviolet light [13] This destroys both codehydrogenases rapidly [133, 134] Codehydrogenase II is unstable in alkaline solution, but stable in acid solution [135]

In isolated muscle tissue codehydrogenase II is rapidly inactivated [136] Codehydrogenase II is optically active $[\alpha]_{589\,m\mu} = -24\ 6°$, $[\alpha]_{546\,m\mu} = -29\ 4°$

**Constitution** Codehydrogenase II has not yet been isolated in the pure form The probable empirical formula is $C_{21}H_{8}N_7O_{17}P_3$ [137] This corresponds to 1 mol adenine, 1 mol nicotinamide, 2 mols pentose (probably *d* ribose) and 3 mols phosphoric acid Codehydrogenase II seems thus to differ from codehydrogenase I only by one additional phosphoric acid group Adenine and nicotinamide have been isolated from the break down products of this coenzyme [138, 131] Codehydrogenase II is dibasic since the results of electrophoresis determinations established two different dissociation constants $p_{K_1} = 1\ 8$ and $p_{K_2} = 6\ 1$ The following formula for codehydrogenase II has tentatively been suggested [140]




131 O Warburg and W Christian *Helv Chim Acta* 19 E 79 (1936) H v Euler E Adler and H Hellström *Z physiol Chem* 241 239 (1936) K Myrbäck H v Euler and H Hellström *Ibid* 212 7 (1932)

133 O Warburg and W Christian *Biochem Z* 282 221 (1936)

134 J Runnström and L Michaelis *J Gen Physiol* 18 717 (1935)

135 O Warburg W Christian and A Griese *Biochem Z* 282 157 (1935)

136 H v Euler H Heiwinkel and F Schlenk *Z physiol Chem* 247 IV (1937)

137 O Warburg W Christian and A Griese *Biochem Z* 282 157 (1935)

138 O Warburg and W Christian *Helv Chim Acta* 19 E 79 (1936) H v Euler E Adler and H Hellstrom *Z physiol Chem* 241 239 (1936) K Myrbäck H v Euler and H Hellström *Ibid* 212 7 (1932)

139 O Warburg and W Christian *Biochem Z* 275 464 (193 )

140 H v Euler and F Schlenk *Z physiol Chem* 246 64 (193 )

No definite proof for this structure has as yet been obtained As a matter of fact this structure seems doubtful in view of the fact that an apparent synthesis of codehydrogenase II from codehydrogenase I by two different methods has been accomplished (for details see below) Since these synthetic methods consist in the addition of one mol of phosphoric acid to codehydrogenase I, it would appear that the proposed formula for the coenzyme II containing three phosphoric acid groups in one chain is rather improbable It may be that one of the phosphoric acid radicals is attached to the molecule of codehydrogenase I as a side chain, thus forming the molecule of codehydrogenase II It is noteworthy that codehydrogenase II has apparently no free amino group since it does not react with nitrite [141]

**Synthesis** It has already been pointed out in the section on Synthesis of Codehydrogenase I (p 233) that a partial synthesis of codehydrogenase II can be accomplished from nicotinic acid or nicotinamide by the action of nucleated cells *in vitro*

Codehydrogenase II can apparently be synthesized from codehydrogenase I since the product obtained shows the same properties as does codehydrogenase II in the test for dehydrogenating Robison ester Ultimate proof for the accomplished conversion is still lacking This assumed conversion of codehydrogenase I into codehydrogenase II has been carried out (1) by means of phosphorus oxychloride in ether,[142] (2) by enzymatic phosphorylation [143]

**Determination by Physical and Chemical Methods** The same methods which were described for the determination of codehydrogenase I can also be applied to codehydrogenase II There is no physical or chemical method of differentiating between these two coenzymes

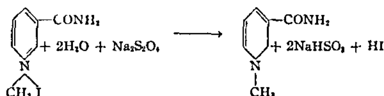
**Determination by Biochemical Methods** 1 Codehydrogenase II and its dihydro form are usually determined according to the Warburg technic by comparison with a standard preparation of this coenzyme in a system which dehydrogenates hexose monophosphoric acid (Robison ester) In addition to the coenzyme, the specific apoenzyme and the yellow ferment are also necessary

**Determination by Biological Methods** Codehydrogenase II can be determined by the growth test with *Haemophilus parainfluenzae* as described for the determination of codehydrogenase I

[141] O Warburg W Christian and A Griese *Biochem Z* 282 157 (1935)
[142] F Schlenk *Naturwissenschaften* 25 668 (1937)
[143] R Vestin *Ibid* 25 667 (1937) H v Euler and R Vestin *Ark Kemi Mineral Geol* B12 No 44 (1938) H v Euler and E Bauer *Ber* 71 411 (1938) H v Euler and E Adler *Z physiol Chem* 252 41 (1938)

## 12 Mechanism of the Nicotinamide Coenzyme Action

It has previously been stated that the action of the various nicotinamide containing enzyme systems consists in the dehydrogenation of various substrates. During this reaction, the codehydrogenase absorbs two atoms of hydrogen, thus being reduced to a dihydro form. The dihydro codehydrogenase is in turn oxidized, and thus reconverted into the codehydrogenase. The nicotinamide part of the molecule is responsible for the phenomenon of the reversible reduction oxidation reaction of the codehydrogenases.[144] *In vitro* this reduction to the dihydro codehydrogenases can be brought about by the action of sodium hydrosulfite $Na_2S_2O_4$ in weakly alkaline solution. In order to study the mechanism of this reaction and the constitution of the reduced and oxidized forms of the coenzymes the reversible reduction of a number of simple nicotinamide derivatives has been examined.[145] It was found that only those derivatives which have a pentavalent ring nitrogen yield dihydro compounds with the same characteristic absorption spectrum as the dihydro coenzyme. Upon reduction the nitrogen becomes trivalent. Nicotinamide iodomethylate proved to be the most characteristic model substance.



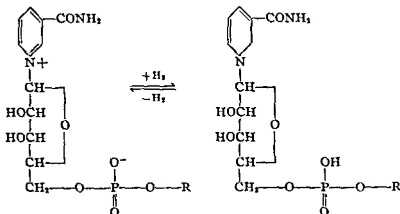

The dihydro nicotinamide compounds but not the corresponding unreduced molecules exhibit a typical whitish fluorescence upon irradiation with ultraviolet light. The dihydro compounds show an additional typical absorption band in the ultraviolet with a maximum at 340 mμ. *By comparison of these characteristics with known dihydro pyridine compounds* it has been concluded that the constitution of the dihydro compound is that of an *o* dihydro compound. Additional evidence is furnished by the deep yellow color of the dihydro compound which would be improbable for a *p* dihydro compound. The results of these experiments indicate that in the natural codehydrogenases the ring nitrogen of nicotinamide is chemically bound in such a way that upon reduction a tertiary

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[144] O Warburg, W Christian and A Griese, *Biochem Z* 282, 157 (1935)

[145] P Karrer and O Warburg, *Ibid* 225, 297 (1936); P Karrer, G Schwarzenbach, F Benz and U V Solmssen, *Helv Chim Acta* 19, 811 (1936); P Karrer and F Benz, *Ibid* 19, 1028 (1936); P Karrer, B H Ringier, J Buchi, H Fritzsche and U V Solmssen, *Ibid* 20, 55 (1937); P Karrer, G Schwarzenbach and G E Utzinger, *Ibid* 20, 72 (1937)

nitrogen is formed The formula of the codehydrogenases is therefore written in the form of a quaternary pyridinium salt which in the dihydro form possesses an additional acidic group



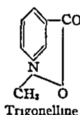

The exact position of the reduced double bond in relation to the carboxylic acid amide group in the dihydro nicotinamide is still unknown It is generally assumed that the double bond in $\alpha\,\beta$ position is reduced the $\gamma\,\delta$ position however cannot be excluded on the basis of the present knowledge [146]

## 13 Specificity of Nicotinic Acid and Nicotinamide

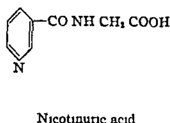
The compounds of this group which are known to be effective when given orally are nicotinic acid its salts for example the sodium salt nicotinamide the *N* methyl amide and the *N* diethyl amide of nicotinic acid ethyl nicotinate nicotinamide glucosido iodide and nicotinuric acid [148] It is assumed as would be expected on a chemical basis that all these compounds are converted in the organism into nicotinamide Since a number of other *N* substituted nicotinamides have been found quite effective in the treatment of heart weakness it is suspected that these derivatives will also prove effective as vitamins

The ring nitrogen apparently must be unsubstituted for the exhibition of vitamin activity, since trigonelline the nicotinic acid methyl betaine is inactive [147] This is easily understood since in enzyme systems the ring nitrogen is attached to the rest of the enzyme molecule

[146] P Karrer F W Kahnt R Epstein W Jaffé and T Ishii *Helv Chim Acta* 21 223 (1938)
[147] D W Woolley F M Strong R J Madden and C A Elvehjem *J Biol Chem* 124 715 (1938)
[148] R W Vilter and T D Spies *Lancet* II 423 (1939)




Trigonelline




Nicotinuric acid

Quinolinic acid,[143] pyrazine carboxylic acid and pyrazine 2,3 dicarboxylic acid are active in curing human pellagra[149] [150] (500–800 mg daily *per os*) and are said not to produce the vasodilator symptoms which often follow the administration of nicotinic acid Dysentery bacilli can use thiazole 5 carboxylic acid as a substitute for nicotinic acid [151] All these compounds were found inactive in dogs [147] [152]

## 14 Determination of Nicotinic Acid and Nicotinamide

### (a) *Chemical Methods*

1 **The Cyanogen Bromide Method** This method is based on the principle[153] that pyridine derivatives give specific colors with cyanogen bromide and a primary or secondary amine In order to determine the quantity of nicotinic acid in natural products, the latter must be totally hydrolyzed to contain only the free acid The solutions, protected from light, are warmed with CNBr in the dark and treated with an amine [154] for example, with *p* amino acetophenone,[155] *β* naphthylamine,[156] aniline,[157] [158] [159] *N* methyl *p* amino phenol [160] *p* methyl amino phenol sulfate[160] or others A yellowish green color develops which is measured in one of the usual apparatus and can be extracted with amyl alcohol [154] The color can be stabilized by a phosphate buffer of pH 6 1 [161]

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[149] C E Bills F G McDonald and T D Spies *Southern Med J* 32 793 (1939)
[150] T D Spies A A Walker and A W Woods *J Am Med Assoc* 113 1481 (1939)
[151] F C Schmelkes *Science* 90 113 (1939)
[152] H A Waisman O Mickelsen J M McKibbin and C A Elvehjem *J Nutrition* 19 483 (1940)
[153] W König *J prakt Chem* 69 105 (1904) 70 19 (1904)
[154] M Swaminathan *Nature* 141 830 (1938) H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938) G E Shaw and C A Macdonald *Quart J Pharm Pharmacol* 11 380 (1938) E Bandier and J Hald *Biochem J* 33 264 (1939)
[155] L J Harris and W D Raymond *J Soc Chem Ind* 58 652 (1939)
[156] H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 208 (1938)
[157] H Kringstad and T Naess *Naturwissenschaften* 43 709 (1938) *Z physiol Chem* 260 108 (1939)
[158] M Swaminathan *Nature* 141 830 (1938) *Indian J Med Research* 26 427 (1938)
[159] D Melnick and H Field *J Biol Chem* 134 1 (1940) 135 53 (1940)
[160] E Bandier and J Hald *Biochem J* 33 264 (1939) E Bandier *Ibid* 33 1130 (1939)
[161] H Kringstad and T Naess *Naturwissenschaften* 26 709 (1938) *Z physiol Chem* 260 108 (1939)

It has been stated that the color developed with *p* amino acetophenone is more suited for quantitative determinations of minute amounts of nicotinic acid than the color from any of the other investigated amines About 1 mg of nicotinic acid in a 1 g sample can be recognized by this procedure [162]

The cyanogen bromide method is not specific for nicotinic acid A number of other pyridine derivatives and especially derivatives of nicotinic acid such as trigonelline nicotinuric acid and nicotine give similar color tests [163]

2 **The 2,4-Dinitro-chlorobenzene Method** [164] The material to be analyzed according to this method should contain only the free nicotinic acid or its amide In most cases therefore, at least a partial hydrolysis must be carried out The dry material is then fused with 2,4 dinitro chlorobenzene and the reaction product is dissolved in alcohol and potassium hydroxide is added The color developed is measured colorimetrically [165, 166]

Neither of these two methods is specific for nicotinic acid as the same colors are given by many pyridine derivatives During systematic studies of the nicotinic acid content of various plant and animal materials it has been observed that the materials especially cereals give color reactions with these two methods in amounts which cannot be explained on the basis of the amount of the nicotinic acid actually present [167] Further inaccuracies arise from procedures in which nicotinic acid is extracted from other undesirable compounds and from attempts to prepare colorless extracts as is necessary for the determination of nicotinic acid in urine and in blood (see p 247)

For the determination of nicotinic acid in tablets and in ampules a tentative A O A C method (Association of Official Agricultural Chemists) has been worked out[168] which consists in subliming preferentially the nicotinic acid out of the sample and by weighing or titrating[169] the sublimate

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[162] I Kodicek *Biochem J* 34 712 7.4 (1940)

[163] D Melnick W D Robinson and H Field *J Biol Chem* 136 131 (1940)

[164] E Vongerichte *Ber* 32 2571 (1899)

[165] P Karrer and H Keller *Helv Chim Acta* 21 463 1170 (1938) S P Vilter T D Spies and A P Mathews *J Biol Chem* 125 85 (1938) *J Am Chem Soc* 60 731 (1938)

[166] H v Euler F Schlenk L Melzer and B Högberg *Z physiol Chem* 258 212 (1939) P Karrer and H Keller *Helv Chim Acta* 22 129 (1939)

[167] F Kodicek *Biochem J* 34 712 724 (1940)

[168] *Methods of Analysis* Assoc Official Agricultural Chemists 1940 p 610 *J Assoc Official Agr Chem* 23 58 (1940)

[169] P S Jorgensen *J Assoc Official Agr Chem* 23 765 (1940)

*Nicotinic acid is separated from its amide* by ether extraction of a water solution Only the amide is extracted by ether

### (b) Biochemical Methods

**Lactobacillus Test** In this test the amount of lactic acid produced by the *Lactobacillus arabinosus* is measured When adequately supplied with all necessary growth factors, the quantity of lactic acid produced is directly proportional to the amount of nicotinic acid present [170] This method has several advantages over the chemical methods of nicotinic acid assay Turbidity and color of the sample to be tested do not interfere with the determination Nicotinic acid and its amide have equal activities The sensitivity of this test is greater than that of the chemical tests One $\gamma$ or less can be determined accurately

### (c) Biological Methods

None of the proposed biological methods listed below has been used extensively enough to claim quantitative determinations under all conditions In earlier qualitative experiments dogs proved very valuable as test animals and it seems that some of the microorganisms will serve in the future for quantitative determinations The following methods have been suggested

1 *Dog test* [171, 172, 173, 174]
2 Chick test [172]
3 Moth test (*Galleria melonella*) [175]
4 Microorganism growth tests on *Staphylococcus aureus* [176, 177, 178] *Shigella paradysenteriae*[179] and *Bacterium proteus* [180]

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170 E E Snell and L D Wright *Proc Am Soc Biol Chem* 1941 CXIX
171 C A Elvehjem R J Madden F M Strong and D W Woolley *J Am Chem Soc* 59 1767 (1937) *J Biol Chem* 123 137 (1938)
172 C J Koehn and C A Elvehjem *J Biol Chem* 118 693 (1937)
173 J Goldberger G A Wheeler R D Lillie and L M Rogers *U S Pub Health Service Pub Health Repts* 43 1385 (1928)
174 W H Sebrell *Ibid* 49 754 (1934) W H Sebrell G A Wheeler and D J Hunt *Ibid* 50 1333 (1935)
175 D Rubinstein and L Shekun *Nature* 143 1064 (1939)
176 M Landy *Ibid* 142 618 (1938)
177 B C J G Knight and H McIlwain *Biochem J* 32 1241 (1938)
178 B C J G Knight *Ibid* 31 731 (1937)
179 H F Fraser N H Topping and W H Sebrell *U S Pub Health Service Pub Health Repts* 53 1836 (1938)
180 A Lwoff and A Querido *Compt rend soc biol* 129 1039 (1938)

### 15 Standard of Nicotinic Acid and Nicotinamide

Up to the present time no national or international standard has been set up for nicotinic acid or nicotinamide There is actually little need for such a standard since nicotinic acid and its amide are well known crystallized compounds which are used on the weight basis

### 16 Physiology of Plants and Microorganisms

Nicotinic acid amide is a normal cell constituent and growth factor for plants and microorganisms It is generally assumed that the coenzyme function in reversible oxidation reduction systems represents the main activity of nicotinic acid but some evidences have been brought forward which indicate that nicotinic acid or its amide may have an additional function outside the coenzyme linkages (see page 245)

The actual need of plants for nicotinic acid has been demonstrated on isolated pea embryos and isolated pea radish and cosmos roots [181, 182] Among the microorganisms are certain species which synthesize their own nicotinic acid requirements while others depend upon an external supply of this essential nutrient Among the organisms which need nicotinic acid as a regular food constituent are certain bacteria such as *B diphtheriae* [183] *B dysenteriae* [184] *B proteus* [185] *Staphylococcus aureus* [186, 187, 188] lactic acid bacteria [189] protozoa such as Protista[188] and certain fungi [188] Still more parasitic organisms need an external supply of the codehydrogenases for proper growth which is not induced by the administration of the components of the coenzyme The *B influenzae* belongs to this group [190]

### 17 Animal Physiology

*General Physiology Metabolism and Mechanism of the Vitamin Action*

The normal dietary intake of nicotinic acid consists mostly of the coenzymes which are present in food of plant and animal origin It is assumed that the enzyme complex is split in the intestinal tract but it is uncertain if the coenzymes can be absorbed as such or if they are hy

J Bonner *Pl t Phys l* 13 865 (1938) F T Addicott and J Bonner *Science* 88 5? (1938)
J Bonner *Am Chem Soc Div Ag F od Chem Meet g* Sept 1939 Abst 13 14
J H Mueller *J Bact* 34 429 (1937) *J Biol Chem* 120 219 (1937)
[184] S A Koser A Dorfman and F Saunders *Proc Soc Exptl Biol Med* 38 311 (1938) ? ? 90 544 (1938)
[185] P Fildes *Brit J Exptl Path* 19 239 (1938)
[186] B C J G Knight *Biochem J* 31 741 (1937)
[187] M Landy *Nature* 142 618 (1938)
[188] B C J G Knight and H M Illwa? *Biochem J* 32 1241 (1938)
[189] E E Snell F M Strong and W H Peterson *J Am Chem Soc* 60 282 (1938)
[190] R W Vilter S P Vilter and T D Spies *J Am Med Assoc* 112 420 (1939)

drolyzed prior to the absorption Nicotinic acid and its amide are absorbed unchanged Nicotinic acid is amidated in the organism after absorption by the blood stream The nicotinic acid and its amide are transported in the blood serum, which maintains a certain level of this essential compound, but contains no coenzymes The blood corpuscles, on the other hand, contain relatively high concentrations of the coenzymes but no free nicotinamide

There are no special storage organs for nicotinic acid or any of its derivatives In the form of the coenzymes, nicotinic acid is present in practically all cells While nicotinic acid deficiency in the dog and pig results in a lowered coenzyme content of the liver and muscles [191, 192, 193] no substantial effect has been noted upon the coenzyme content of the brain, kidney cortex and blood In human beings nicotinic acid deficiency causes a marked decrease in the coenzyme content of striated muscles but has only a slight effect upon the coenzyme content of the erythrocytes [194]

Nicotinic acid is, like all the other vitamins, secreted in milk and is found in eggs

Nicotinic acid and its metabolic end products are excreted through the urine The normal human organism excretes a certain amount in the free form Coenzymes are not excreted [195, 196] Ingested nicotinic acid is excreted in considerable amounts in the form of nicotinuric acid and trigonelline There is, furthermore, apparently a combined form of nicotinic acid in the urine[197] which may be different from nicotinuric acid The total output depends upon the intake Normally about 4–5 mg are excreted daily [198] Lowered values have been observed in pellagrins and during times of anorexia Guinea pigs deprived of nicotinic acid show a progressive decrease of nicotinic acid excretion which reaches a zero value at the time when clinical deficiency symptoms develop Dogs excrete only trigonelline and nicotinuric acid but no nicotinic acid or its amide [199] Rabbits, on the other hand cannot synthesize trigonelline from nicotinic acid [200]

Nicotinic acid appears to function mainly as a part of enzyme systems which have been discussed previously and which take part in the protein

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[191] A E Axelrod R J Madden and C A Elvehjem *J Biol Chem* 131 85 (1939)
[192] H I Kohn J R Klein and W J Dann *Biochem J* 33 1432 (1939)
[193] M Pittman and H F Frazer *U S Pub Health Service Pub Health Repts* 55 915 (1940)
[194] A E Axelrod T D Spies and C A Elvehjem *J Biol Chem* 138 667 (1941)
[195] R W Vilter S P Vilter and T D Spies *J Am Med Assoc* 112 4_0 (1939)
[196] H v Euler F Schlenk H Heiwinkel and B Högberg *Z physiol Chem* 256 ?08 (1938)
[197] E Bandier *Biochem J* 33 1787 (1939)
[198] L J Harris and W D Raymond *J Soc Chem Ind* 58 652 (1939)
[199] D Ackermann *Z Biol* 59 17 (1912)
[200] W A Perlzweig H P Sarett and J W Huff *Proc Am Soc Biol Chem* 1941 C

and the carbohydrate metabolism by transporting hydrogen. At birth the tissues of mammals contain only small amounts of the nicotinamide coenzymes I and II (about 100–150 γ per gram of liver or kidney in rats), but the amount rises rapidly and reaches the normal range of adults (about 550 γ in rats) in about seven days.[201]

There is the possibility that nicotinic acid may act in other functions than through the coenzymes I and II. Thus nicotinic acid deficiency causes water retention and may therefore be linked with the water metabolism.[202] This effect could, however, be understood as a side reaction of the disturbance of the carbohydrate metabolism. Nicotinic acid also influences the metabolism of the heavy metals. Also in this case as in the influence upon the water metabolism, no decision can be made on the basis of the experimental facts as to whether this is a primary action of nicotinic acid, its amide or the coenzymes or if these disturbances are secondary reactions. A pigmentation in the skin and the mucous membranes of the mouth is often observed in a human being suffering from a nicotinic acid deficiency. The pigment is not melanin but an iron pigment.[203] As the result of a nicotinic acid deficiency a type of anemia occurs which responds favorably to iron therapy. The iron containing porphyrins undergo some kind of decomposition and as the result of this an increased amount of porphyrin compounds is secreted through the urine.[204] The copper and iron containing enzyme systems such as cytochrome, the polyphenol oxidases, the peroxidase and catalase appear to be affected. Thus for example a disturbance of the tryptophane catabolism has been observed in pellagrins which resulted in the excretion of indoxyl ethyl amin.[205] The disturbances of the sympathetic nervous system could perhaps be explained as a disturbed relation of the polyphenol oxidases to adrenalin.

Nicotinic acid decreases the peristaltic action of the stomach and small intestine while inositol (see page 279) increases this action. The other members of the B complex have no apparent action of this type. It has therefore been suggested that the balance or ratio of nicotinic acid to inositol is the nutritional factor which determines hypo or hyper gastrointestinal motility.[206]

There are observations which indicate functions of nicotinic acid which cannot be explained on the basis that nicotinic acid acts only through the

[201] F. Bernheim and A. v. Felsovanyi, *Science* 91, 76 (1940).
[202] C. Funk and I. C. Funk, *Z. Vitaminforsch.* 8, 330 (1938).
[203] H. Herzenberg, *Beitr. path. Anat.* 96, 37 (1935).
[204] T. D. Spies, W. B. Bean and R. F. Stone, *J. Am. Med. Assoc.* 111, 584 (1938).
[205] M. X. Sullivan, *J. Biol. Chem.* 50, 39 (19  ).
[206] G. J. Martin, M. R. Thompson and J. de Carvajal Forero, *Am. J. Digest. Dis.* 8, 290 (1941).

coenzymes Blacktongue in dogs can be cured easily by nicotinic acid, but no significant effect could be noted when cozymase was injected intravenously in an amount which on the basis of its nicotinic acid content would have been expected to exert beneficial effects[206] Growth and respiration of dysentery bacilli are much more favorably influenced by nicotinic acid or its amide than by the coenzymes, and the efficacy of the action of the coenzymes can be increased markedly by hydrolysis under conditions which free the nicotinamide[207, 208]

## 18 Avitaminosis

There are no clinical symptoms known for slight nicotinic acid deficiency in man Definite diagnostic evidences develop late[209] In severe cases the blood level of nicotinic acid is decreased This has frequently been observed[210] in pregnant women on diets low in nicotinic acid, since during pregnancy increased amounts of this vitamin are needed Another relatively early symptom of nicotinic acid deficiency is the excretion of porphyrins in the urine.[211]

The typical symptoms of nicotinic acid deficiency in man are commonly summarized in the term pellagra The typical pellagrin shows characteristic lesions of the mucous membranes, for example in the mouth (glossitis), and of the skin over the nose forehead, dorsum hands wrists elbows knees and feet This type of dermatitis involves especially those parts of the body which are exposed to sunlight or to friction Disturbances of the gastrointestinal tract are observed in many cases In later stages of the disease, mental disorders and lesions of the central nervous system occur, which are characterized by clouding of consciousness cogwheel rigidities and uncontrollable grasping and sucking reflexes[1] A certain form of anemia has been observed in severe cases Diagnosis of pellagra is sometimes very difficult since the symptoms described do not necessarily occur all at once

Typical pellagra is, however, not a disease caused solely by a nicotinic acid deficiency Usually pellagra is the result of a multiple vitamin deficiency and can be cured only by the administration of several or all

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207 F S Daft H F Frazer W H Sebrell and M Pittman *Science* 88 128 (1938)

208 A Dorfman S A Koser H R Reames K F Swingle and F Saunders *Proc Soc Exptl Biol Med* 43 163 (1940) F Saunders A Dorfman and S A Koser *J Biol Chem* 138 69 (1941) L C Norris and A T Ringrose *Science* 71 643 (1930)

209 M A Blankenhorn and T D Spies *J Am Med Assoc* 108 583 (1937)

210 A Lwoff A Querido L Digonnet and Carmier *Compt rend soc biol* 131 900 (1939)

1 T D Spies W B Bean and R F Stone *J Am Med Assoc* 111 584 (1938)

211 N Jolliffe K M Bowman L A Rosenblum and H D Fein *Ibid* 114 307 (1940)

members of the vitamin B complex Besides nicotinic acid, riboflavin and thiamin therapy are especially necessary [213, 214]

Nicotinic acid treatment has been observed to give beneficial results to pneumonia patients who under sulfanilamide therapy have developed a black dotted heavy furring of the tongue which resembles the blacktongue of dogs [215]

The lesions in the mouth caused by nicotinic acid deficiency sometimes are very similar in appearance to the lesions of Vincent's infection but are genetically different It seems that Vincent's infection frequently sets in when the mucous membranes are weakened due to nicotinic acid deficiency and in such cases a treatment with nicotinic acid is, of course, beneficial [216] Encouraging results have been obtained by administration of nicotinic acid for the prevention and treatment of irradiation (x ray) sickness [217] Furthermore it has been suggested[218] that nicotinic acid may be of value in the treatment of eighth nerve (high tone) deafness

In dogs the typical syndrome of nicotinic acid deficiency is the occurrence of blacktongue In adrenalectomized rats a special form of dermatitis has been noted which responds favorably to treatment with nicotinic acid [219]

## (a) *Clinical Test Methods*

The methods which have been suggested for the determination of a state of nicotinic acid deficiency comprise the determination of the amount excreted in urine or present in blood In addition, the occurrence of porphyrins in the urine can be used as an additional test method

**Urine Tests** The determination of the excretion of nicotinic acid is difficult since urine contains this compound not only in the free form but also in the form of various derivatives mainly as nicotinamide, nicotinuric acid and trigonelline In order to obtain uniform results, the urine must be hydrolized, preferably with alkali [220] The color present in urine invariably interferes with colorimetric determinations for the nicotinic acid content Preferential adsorption of the colored materials on

[213] G Margolis L H Margolis and S G Smith *J Nutrition* 16 541 (1938) 17 63 (1939) O M Helmer and P J Fouts *Ibid* 16 271 (1938) W H Sebrell *J Am Med Assoc* 110 1665 (1938) R W Vilter S P Vilter and T D Spies *Ibid* 112 420 (1939)

[214] R W Vilter S P Vilter and T D Spies *J Am Med Assoc* 112 4 0 (1939)

[215] F M Josephson and G Klewan *Nature* 143 7?? (1939)

[216] R Sophi *Am J Digestive Diseases Nutrition* 7 298 (1940) J D King *Lancet* 2 32 (1940)

[217] J W Cr ham *J Am Med Assoc* 113 664 (1939)

[218] G Selfridge *Ann Otol Rhin Laryng* 48 39 (1939)

[219] L Laszt *Z Vitamins f rsch* 11 76 (1941)

[220] F Bandier *Biochem J* 33 1787 (1939)

charcoal or on zinc hydroxide[221] has been suggested None of the color reactions can be considered reliable since urine contains substances which interfere with the determination As the result of these difficulties, only approximate values can be expected from the determination of nicotinic acid in urine For the actual test, the *color reaction with 2,4-dinitro chlorobenzene* has been modified for urine analysis[222] The hydrolyzed and decolorized urine is evaporated and the dry residue is mixed with 2,4 dinitro chlorobenzene in alcohol, and after standing for several hours the alcohol is distilled off and the residue is heated to 105° C for 10 minutes After cooling, a solution of KOH in alcohol is added A purple color develops which can be determined by the usual methods The *cyanogen bromide method* has frequently been used for the determination of nicotinic acid in urine and a number of slight modifications of the basic technic have been recommended[223, 224, 225, 226] Both of these methods are unreliable because other compounds, for example, nicotine and trigonelline, give similar color reactions[227]

A somewhat better method for the determination of a deficiency is to perform an *excretion test* on the patient[228] Excess doses of nicotinic acid or its amide are given and the amount excreted is determined before and after the ingestion The total amount of compounds which behave like nicotinic acid in the color tests varies considerably in normal persons[229] and is dependent on many factors Thus, smokers usually excrete increased amounts of chromogenic material Upon oral administration of, for example 500 mg of nicotinic acid to normal individuals, the urinary output rises to its maximum within one hour and drops to normal in about four hours In a series of experimental tests it was found that from the ingested 500 mg about 110 mg are excreted by normal persons Of this total amount about 51% is present as trigonelline, 36% as nicotinuric acid and 13% as free nicotinic acid or nicotinamide Coenzymes are normally not excreted

A state of nicotinic acid deficiency can also be suspected from the *excretion of porphyrins through the urine*, but it should be remembered that

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221 T E Friedemann and C J Barborka *J Biol Chem* 138 787 (1941)
222 P Karrer and H Keller *Helv Chim Acta* 21 463 1170 (1938) S P Vilter T D Spies and A P Mathews *J Biol Chem* 125 85 (1938) *J Am Chem Soc* 60 731 (1938)
223 E Bandier *Biochem J* 33 1787 (1939)
224 L J Harris and W D Raymond *Ibid* 33 2037 (1939)
225 D Melnick and H Field *J Biol Chem* 134 1 (1940)
226 L A Rosenblum and N Jolliffe *Ibid* 134 137 (1940)
227 D Melnick W D Robinson and H Field *Ibid* 136 131 (1940)
228 D Melnick W D Robinson and H Field *Ibid* 136 145 (1940)
229 D Melnick W D Robinson and H Field *Ibid* 136 157 (1940)

porphyruria may have other causes than a nicotinic acid deficiency In the actual test, the urine is acidified with acetic acid and extracted with ether The ether solution is washed with water and then with 25% hydrochloric acid The latter causes the appearance of a color, which indicates the presence of porphyrins [230]

Blood Tests Blood contains both nicotinic acid (or its amide) and the codehydrogenases The latter are almost completely confined to the blood corpuscles [231, 232, 233] while the serum contains only the free nicotinic acid (or its amide) Of the total amount about 90% is in the erythrocytes and this amount decreases only very slightly at times of nicotinic acid deficiency The small amount of free nicotinic acid present is a function of the intake Upon oral administration of nicotinic acid to fasting persons the blood level increases promptly but returns rapidly to values only slightly higher than the basal level [232] The total nicotinic acid content of the blood of normal persons ranges between 0 52 and 0 83 mg % [233]

The actual determination of the nicotinic acid (or its amide) can be carried out by means of the *cyanogen bromine method*,[234, 235, 236, 237, 238] by the *Bacillus proteus growth method*[239] and the codehydrogenase I can be determined by the *fermentation method* [240]

## 19 Hypervitaminosis

The intake of about 1000 times the amount of nicotinic acid which is normally consumed in food may be considered as relatively non toxic Higher doses however exhibit typical toxicological symptoms Dogs kept at a daily intake of 2 g of nicotinic acid died within twenty days In human beings the oral administration of large quantities of nicotinic acid is followed by flushing, burning itching and increased sensations of local heat in the skin Nicotinic acid amide does not produce these symptoms [241] and is therefore recommended for clinical use

[230] T D Spies R W Vilter and W F Ashe *J Am Med Assoc* 113 931 ([illegible])
[231] A Dorfman M K Horwitt S A Koser and F S under *Proc Am Soc Biol Chem J Biol Chem* 128 XX (1939)
[232] H I Kohn and J R Klein *J Biol Chem* 130 1 (1939)
[233] D Melnick W D Robinson and H Field *Ibid* 136 157 (1940)
[234] D Melnick and H Field *Ibid* 134 1 (1940) 135 53 (1940)
M Swaminathan *Indian J Med Research* 26 427 (1938)
[236] P B Pearson *J Biol Chem* 129 491 (1939)
[237] K Ritsert *Klin Wochschr* 18 934 (1939)
[238] T D Spies A A Walker and A W Woods *J Am Med Assoc* 113 1481 (1939)
[239] A Querido M Albeaux Fernet and A Lwoff *Compt rend soc biol* 131 18 (1939) A Querido A Lwoff and C Latast *Ibid* 130 1580 (1939)
[240] A E Axelrod and C A Elvehjem *J Biol Chem* 131 77 (1939)
[241] T D Spies, D P Hightower and L H Hubbard *J Am Med Assoc* 115 29 (1940)

## 20 Nicotinic Acid Requirements

It is established that all living tissues need nicotinic acid Some organisms need an external supply of this vitamin while others are able to synthesize it

The daily requirements of man are of the order of 12–23 mg (Details see page 613) Therapeutically, nicotinic acid has been given orally in daily doses ranging from 50 to 500 mg Monkeys apparently need about 5 mg daily [242] The necessity of a regular intake of nicotinic acid has also been proved for dogs,[243] pigs [244] and the moth *Galleria melonella* [245]

Certain species of microorganisms are able to synthesize nicotinic acid such as, for example, *Chilomonas paramecium*,[246] while other species, such as *Bacterium pneumococcus*,[247] need an external supply of nicotinic acid for proper growth [248, 249] Some other unicellular organisms, for example the *Bacillus influenzae*,[250] do not grow upon administration of nicotinic acid or its amide, but need codehydrogenase I or II Some of the microorganisms which are able to synthesize nicotinic acid have been shown to live in the intestinal tract of higher animals, such as, for example in sheep [251] and in cattle [252]

The nicotinic acid requirement of the rat has been the subject of extensive discussion *While it is certain that the rat needs this vitamin* [253, 254, 255] the question as to whether or not the rat must have nicotinic acid in its daily food intake is not settled It has been suggested that the rat is able to synthesize its own supply,[256] but it seems more plausible to assume that if rats do not need to have nicotinic acid in their food, they have microorganisms living in their intestinal tract which synthesize this vitamin and from which the rats obtain the necessary amount

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242 L J Harris *Biochem J* 32 1479 (1938)
243 C A Elvehjem R J Madden F M Strong and D W Woolley *J Am Chem Soc* 59 176 (1937) *J Biol Chem* 123 137 (1938)
244 H Chick T F Macrae A J P Martin and C J Martin *Biochem J* 32 10 (1938)
245 D Rubinstein and L Shekun *Nature* 143 1064 (1939)
246 J O Hutchens B J Jandorf and A B Hastings *J Biol Chem* 138 321 (1941)
247 L Rane and Y Subbarow *Ibid* 134 455 (1940)
248 B C J G Knight *Biochem J* 31 731 (1937)
249 J H Mueller *J Bact* 34 429 (1937) *J Biol Chem* 120 219 (1937)
250 A Lwoff and M Lwoff *Proc Roy Soc (London)* B122 352 360 (1937) *Compt rend* 203 520 (1936)
251 A H Winegar P B Parson and H Schmidt *Science* 91 508 (1940)
252 M I Wegner A N Booth C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 45 769 (1940)
253 D V Frost and C A Elvehjem *J Biol Chem* 121 255 (1937) P György *Proc Soc Exptl Biol Med* 37 732 (1938) W R Wyatt *Iowa State Coll J Sci* 14 103 (1939)
254 H v Euler F Schlenk L Melzer and B Högberg *Z physiol Chem* 258 212 (1939)
255 J Laszt *Z Vitaminforsch* 11 76 (1941)
256 W J Dann and H I Kohn *J Biol Chem* 136 435 (1940)

# PANTOTHENIC ACID

# PANTOTHENIC ACID

## 1 Nomenclature and Survey

### Names

Pantothenic acid[1] (the name is derived from the Greek meaning from everywhere )
Pantothen Abbreviated term
Antidermatosis vitamin[2]
Chick antidermatitis factor[3]
Vitamin B [4]
Filtrate factor (liver filtrate factor[5] yeast filtrate factor[6])
Factor 2 (from liver)[7]
Chick A P factor (Chick Anti pellagra factor)[8] The term pellagra is properly used only in describing human diseases (in compliance with the Committee on Vitamin Nomenclature)
Vitamin $B_2$ (G)[9] (abandoned historical name)

### Probably also identical with

*Vitamin $B_3$* Vitamin $B_3$ is a term given in 1928 to a heat labile factor necessary for weight maintenance of pigeons [10, 11] Recent findings point to the identity of this vitamin with pantothenic acid [12]

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[1] R J Will ams C M Lyman G H Goodyea T H Truesd il and D Holaday *J Am Chem Soc* 55 2312 (1933)

[2] J C Bauernfeind A E Schumacher A Z Hodson L C Norris and G F Heuser *Proc Soc Expll Biol Med* 39 108 (1938)

[3] O Mickelsen H A Waisman and C A El ehjem *J B ol Chem* 124 313 (1938) D W Woolley H A Waisman O M ckelsen and C A Elvehjem *Ibid* 125 715 (1938)

[4] A Bakke V Aschehoug and C Zb nden *Compt rend* 191 1157 (1930) A F Morgan B B Cook and H C Davison *J Nutriti n* 15 7 (1938) A F Morgan and H D Simms *Ibid* 19 233 (1940) G Lunde and H Kringstad *Arch Norske Vid Akad Oslo* I Math Naturw Klasse No 1 1 (1938) *Z physiol Chem* 257 01 (1939) 261 110 (1939) *Naturw sse schaften* 27 755 (1939) *Angew Chem* 52 521 (1939) G Lunde H Kringstad and E Jansen *Naturw ssenschaften* 29 62 (1941)

[5] T H Jukes and S Lepkovsky *J B ol Chem* 111 119 (1935) 114 109 117 (1936)

[6] C E Edgar and T F M crae *Biochem J* 31 886 893 (1937)

[7] S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 5 7 (1936)

[8] C J Koehn and C A Elvehjem *Ibid* 118 693 (1937)

[9] C A Elvehjem and C J Koehn *Nature* 134 1007 (1934) *J Biol Chem* 108 709 (1935) Th se authors suggested that the chick pell gra preventative factor be called vitamin $B_2$ or G in d f ferentiation from riboflav n S nce however this vitamin is now called vitamin $B_2$ or G the utilization of these letters to de gnate pantothen c acid has been abandoned For a discuss on of this nomen cl ture see al o ref 5 and C J Koehn and C A Elvehjem *J Nutrition* 11 67 (1936)

[10] R R Will ams and R E Waterman *J B ol Chem* 78 311 (1928)

[11] C. W Carter and J R O Bri n *Biochem J* 30 43 (1936)

[12] C. W Carter and J R O Brien *Ib d* 33 1810 (1939)

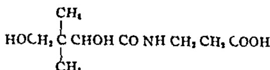
*The spectacled eye condition preventive factor* for rats The spectacled eye condition has been described[13] as an erosion occurring around the eyes of rats and imparting to the animals a spectacled appearance The lids became denuded and scaly In more severe cases the eyes are closed by a sticky exudate Since concentrates of pantothenic acid brought about a rapid cure of the spectacled eye condition it is supposed[14] that the two factors may be identical (See however pages 280 and 477 )

**Empirical formula**



$C_9H_{17}O_5N$

**Structural formula**




**Chemical name**

(+) α γ Dihydroxy β β dimethyl butyryl β′ alanide

**Efficacy**

1 g of pantothenic acid = 70 000–75 000 Chick Units

## 2 Chronology

1930 NORRIS and RINGROSE[15] described a specific dermatosis of chicks

1933 WILLIAMS and co workers[16] found that a naturally occurring compound of unknown chemical composition called *pantothenic acid* stimulates the growth of yeast

1934–1935 ELVEHJEM and KOEHN[17] differentiated the chick antidermatitis factor from riboflavin and cured the chick syndrome with liver extract

1936 LEPKOVSKY JUKES and KRAUSE showed the need of rats for a factor distinct from thiamin riboflavin and vitamin $B_6$ [18]

1937 *FOUTS LEPKOVSKY HELMER and JUKES[19] and independently* DANN[20] stated that nicotinic acid does not cure or prevent chicken dermatitis

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[13] J Goldberger and R D Lillie *U S Pub Health Service Pub Health Repts* 41 1025 (1926) A Bourquin and H C Sherman *J Am Chem Soc* 53 3501 (1931) H E Robinson and R C Newton Abstracts Division of Biological Chemistry Am Chem Soc Kansas City April 13 17 (1936) S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 557 (1936)

[14] J J Oleson H R Bird C A Elvehjem and E B Hart *J Biol Chem* 127 23 (1939)

[15] L C Norris and A T Ringrose *Science* 71 643 (1930) A T Ringrose L C Norris and G F Heuser *Poultry Sci* 10 166 (1931)

[16] R J Williams C M Lyman G H Goodyear T H Truesdail and D Holaday *J Am Chem Soc* 55 2912 (1933)

[17] C A Elvehjem and C J Koehn *Nature* 134 1007 (1934) *J Biol Chem* 108 709 (1935)

[18] S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 557 (1936)

[19] P J Fouts S Lepkovsky O M Helmer and T H Jukes *Proc Soc Expll Biol Med* 37 405 (1937)

[20] W J Dann *Science* 86 616 (1937)

1938 The chemical nature of pantothenic acid was worked out by R J WILLIAMS and co workers [21]

1939 The identity of pantothenic acid and the chick antidermatitis factor was recog nized by Jukes[22] and by WOOLLEY WAISMAN and ELVEHJEM [23]

1940 Pantothenic acid was totally synthesized by STILLER HARRIS FINKELSTEIN KERESZTESY and FOLKERS[24] and independently by REICHSTEIN and GRÜSSNER [25] and by KUHN and WIELAND [26]

## 3 Occurrence

Pantothenic acid occurs in all types of animal tissues and seems to be present universally in protoplasm The liver and kidney are the richest known animal sources of pantothenic acid followed by heart spleen brain pancreas tongue and lung [27] the muscular tissue (of beef lamb pork and veal) contains considerably less Pantothenic acid is produced by various molds and microorganisms[28] and by green plants after they de velop their photosynthetic apparatus The storage organs in plants, for example rice bran appear to be especially rich in pantothenic acid [29] Molasses[30] contains considerably more than normal plant tissue, for ex ample alfalfa [30]

In animal tissue (and probably also in plant tissue) pantothenic acid occurs free only to a very small extent but is usually found chemically bound to protein material [31]

## 4 Isolation

The isolation of pantothenic acid consists for example when using liver as the starting material, of the following steps [32] First the liver is allowed to autolyze in water The water suspension is then heated and filtered from coagulated, inactive material In place of this step alcoholic liver extracts may also be used as starting material By treatment with fuller s earth much inactive, especially basic material is removed from the water

[21] R J Williams H H Weinstock E Rohrmann J H Truesdail H K Mitchell and C E Meyer *J Am Chem Soc* 61 454 (1939)

[22] T H Jukes *Ibid* 61 975 (1939)

[23] D W Woolley H A Waisman and C A Elvehjem *Ibid* 61 977 (1939)

[24] E T Stiller S A Harris J Finkelstein J C Keresztesy and K Folkers *Ibid* 62 1785 (1940)

[25] T Reichstein and A Grüssner *Helv Chim Acta* 23 650 (1940)

[26] R Kuhn and T Wieland *Ber* 73 971 1134 (1940)

[27] H A Waisman O Mickelsen and C A Elvehjem *J Nutrition* 18 247 (1939)

[28] C H McBurney W B Bollen and R J Williams *Proc Natl Acad Sci U S* 21 301 (193 )

[29] R J Williams and R Moser *J Am Chem Soc* 56 169 (1934)

[30] T H Jukes *J Biol Chem* 114 11 (1937)

[31] R Kuhn and C Wendt *Ber* 71 780 (1938)

[32] R J Williams J H Truesdail H H Weinstock E Rohrmann C M Lyman and C H Mc Burney *J Am Chem Soc* 60 2719 (1938)

solution Pantothenic acid is then adsorbed on Norite at a pH of approximately 3 6 and immediately afterwards eluted[33] with ammonium hydroxide solution A second adsorption on Norite may be carried out[33] followed by elution with a mixture of pyridine and methanol

After neutralization with oxalic acid, the brucine salt of pantothenic acid is prepared and is extracted with chloroform The crude brucine salts are fractionated by various distributions between chloroform and water These salts are converted into the corresponding calcium salts by treating in solution with an excess of lime water and freeing from brucine by filtration and repeated extraction with chloroform Purification of the calcium pantothenate consists in the precipitation of the active principle with alcohol, removal of some impurities by precipitation with mercuric chloride, fractional precipitation of the calcium salt with isopropyl ether and fractional precipitation from pyridine solution with acetone

By application of these methods, about 3 g of crude, approximately 40% pure material were obtained from 250 kg of liver[32, 34] A modification[33] of this procedure is to precipitate impurities from the concentrated eluates of the charcoal adsorption with barium hydroxide until a pH of about 8 is reached and to dissolve the precipitated barium salt of pantothenic acid in absolute alcohol After filtration the barium salt of the vitamin is obtained by concentrating the filtrate and by adding acetone, thus precipitating the acetone insoluble barium salts The free acids are obtained from the barium salts by means of sulfuric acid and can be extracted with ether or amyl alcohol Thus a concentrate containing approximately 20–25% pantothenic acid is obtained

A somewhat different method consists[35] in freeing a liver (for example, tuna fish liver) extract from the fat soluble material and precipitating some impurities with mercuric acetate The active material is then adsorbed on charcoal and eluted with a pyridine methanol water mixture Impurities are removed from the eluate by precipitation with phosphotungstic acid and uracil-*d* riboside (uridine) is removed from the concentrated filtrate by crystallization from methanol The pantothenic acid is then precipitated from the methanol solution with barium hydroxide Further purification can be achieved by repeated precipitation (of impurities) with phosphotungstic acid The active compound is then adsorbed on aluminum oxide, which has been activated with hydrochloric acid

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[33] H K Mitchell H H Weinstock F F Snell S R Stanbery and R J Williams *J Am Chem Soc* 62 1776 (1940)
[34] Y Subbarow and G H Hitchings *Ibid* 61 1615 (1939)
[35] R Kuhn and T Wieland *Ber* 73 962 (1940)

## 5 Properties

Pantothenic acid is predominantly of acid character but shows also some basic properties[36, 37] The vitamin is readily soluble in water ethyl acetate, dioxane, glacial acetic acid, etc, somewhat soluble in ether and amyl alcohol[33] and practically insoluble in benzene, chloroform etc[38] The compound is highly hydrophilic and can be adsorbed on charcoal[37] but not on fuller s earth[37, 39] The acetyl derivative can be distilled at approximately $10^{-5}$ mm Hg[33] The vitamin is sensitive toward acids, bases and heat

Pantothenic acid in pure form is a pale yellow viscous oil The vitamin is dextrorotatory showing $[\alpha]_D^{25}$ +37 5° It forms a microcrystalline calcium salt, $[\alpha]_D^{26}$ +24 3°[40]

## 6 Chemical Constitution

Pantothenic acid has a molecular weight of 219 (values obtained experimentally are approximately 200[41, 4]) and has the empirical formula $C_9H_{17}O_5N$[42] The presence of a carboxyl group in the molecule of this vitamin was proved[42] by esterification with both diazomethane and methanol and recovery of the free acid by careful saponification Thus two of the five oxygens of the suggested empirical formula are accounted for Two other oxygens are present in the form of free hydroxyl groups[43] The presence and number of hydroxyl groups are suggested by the hydrophilic nature of pantothenic acid and of its methyl ester The ester is even more soluble in water than in ether Pantothenic acid is esterified by various acids and acid halides and thereby loses the vitamin activity which can be recovered by careful saponification A determination of the active hydrogen atoms suggested the presence of two hydroxyl groups Further evidence for the existence of at least two hydroxyl groups is based upon the observation that pantothenic acid condenses reversibly with acetaldehyde,

[36] R J Williams and D H Saunders *Biochem J* 28 1887 (1934) O W Richard *J Biol Chem* 113 531 (1936)

[37] R J Williams J H Truesdail H H Weinstock E Rohrmann C M Lyman and C H McBurney *J Am Chem Soc* 60 2719 (1938)

[38] D W Woolley H A Waisman O Mickelson and C A Elvehjem *J Biol Chem* 125 715 (1938)

[39] S Lepkovsky T H Jukes and M E Krause *Ibid* 115 5o7 (1936)

[40] E T Stiller S A Harris J Finkelstein J C Keresztesy and K Folkers *J Am Chem Soc* 62 1785 (1940)

[41] R J Williams C M Lyman G H Goodyear T H Truesdail and D Holaday *Ibid* 55 ?912 (1933)

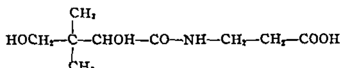
[42] R J Williams H H Weinstock E Rohrmann J H Truesdail H K Mitchell and C E Meyer *Ibid* 61 454 (1939)

[43] R J Williams and R Moser *J Am Chem Soc* 56 169 (1934)

acetone and benzaldehyde This type of reaction suggests the presence of an $\alpha,\beta$, $\alpha,\gamma$ or $\alpha,\delta$ glycol The behavior of pantothenic acid in an electrical field[43] indicated the ionization constant to be approximately $3\,9 \times 10^{-5}$ This corresponds in strength approximately to that of a $\beta$ or $\gamma$ hydroxy-carboxylic acid The absence of a hydroxyl group in $\alpha$ position to the carboxyl group is also suggested by the failure of pantothenic acid to produce a color reaction with ferric chloride

The chemical nature of the fifth oxygen atom and of the nitrogen present in pantothenic acid becomes apparent from hydrolysis experiments Pantothenic acid is inactivated by acids and by alkali From the alkali hydrolysis material, $\beta$ alanine has been isolated[44] and identified as $\beta$ naphthalene sulfo $\beta$ alanine [45] The other part of the molecule is an aliphatic dihydroxy acid which has not been isolated as such In acid solution, especially upon heating, a lactone[46] is readily formed, indicating that a hydroxyl group may be in $\gamma$- or in $\delta$ position to the carboxyl group The former position is indicated by the previously discussed condensation reaction with acetone and other ketones or aldehydes since the condensation would cause the formation of seven membered rings in case the second hydroxyl group was in $\delta$ position

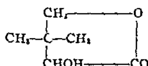
Pantothenic acid has then the following structural formula



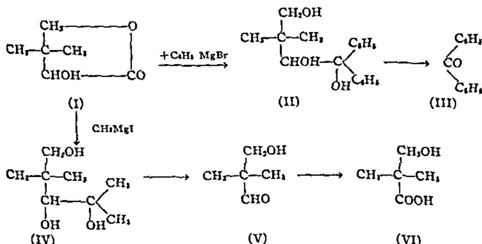

The amino group of the $\beta$ alanine is bound to the carboxyl group of the dihydroxy acid, forming an acid amide group Thus, the nitrogen has been accounted for and it has been shown that there are no amino, imino or tertiary amino groups in the molecule The other hydroxyl group is in $\alpha$ position to the carbonyl group since the acid but not the lactone from the non $\beta$ alanine portion gives[47] a positive ferric chloride test[48] and since carbon monoxide has been obtained[47] by sulfuric acid decomposition of the dihydroxy acid The $\alpha$ $\beta$ positions of the two hydroxyl groups are excluded by the failure[47] of pantothenic acid to react with lead tetra acetate and with periodic acid The lactone has been obtained by ether extraction

[43] D W Woolley H A Waisman and C A Elvehjem *J Am Chem Soc* 61 977 (1939)
[44] H H Weinstock H K Mitchell E F Pratt and R J Williams *Ibid* 61 1421 (1939)
[45] D W Woolley H A Waisman and C A Elvehjem *J Biol Chem* 129 673 (1939)
[47] H K Mitchell H H Weinstock E E Snell S R Stanbery and R J Williams *J Am Chem Soc* 62 1776 (1940)
[48] M A Berg *Bull soc chim* (3) 11 882 (1894)

of acid hydrolyzates as a pure crystalline material[49, 50] and proved to be α hydroxy β β dimethyl γ butyrolactone[50]




Direct titration[51] of this compound showed the absence of a free carboxyl group but on heating with alkali one mol of alkali was consumed proving the presence of a lactone ring The lactone contains one active hydrogen atom and one hydroxyl group since a mono acetate was obtained upon acetylation Oxidation[51] of the lactone with cold alkaline barium hydroxide yielded acetone which indicates the presence of a dimethyl group This was furthermore shown by a Kuhn Roth determination of $CH_3$ side chains Upon reaction with phenyl magnesium bromide the lactone yields a diphenyl carbinol (II) which upon treatment with lead tetra acetate forms benzophenone (III) Methyl magnesium iodide when reacted with the lactone, forms a dihydroxy dimethyl carbinol (IV), which upon oxidation with lead tetra acetate forms the aldehyde (V) The latter on oxidation with alkaline silver hydroxide yields α α dimethyl β hydroxy propionic acid (VI), thus proving the structure of the complete carbon skeleton By comparing the lactone with synthesized material it was shown to be the laevorotatory form[51]




The α γ dihydroxy β β dimethyl butyric acid is dextrorotatory

49 D W Woolley *Science* 91 245 (1940)
50 R J Williams and R T Major *Ibid* 91 246 (1940)
51 F T Stiller J C Keresztesy and J Finkelstein *J Am Chem Soc* 62 1779 (1940)

## 7 Synthesis

At a time when the structure of pantothenic acid was unknown, a hemi synthesis was achieved[52, 53] by condensation of synthetic β alanine ethyl ester with the dihydroxy carboxylic acid isolated from the hydrolysis products of pantothenic acid The dihydroxy acid was acetylated and converted into the acid chloride for utilization in the condensation After careful saponification of the condensation product pantothenic acid was obtained

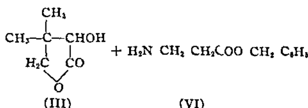
Since the utilization of an acetylated acid chloride is somewhat difficult and gives low yields another method has been worked out This consists in the condensation of an ester of β alanine with the lactone of the dihydroxy acid[54, 55, 56] Yields up to 50% are obtained by using this procedure

An improved method, both simple and effective, is the direct condensation[54] of β alanine (IV) with the lactone (III) This method has the advantage of avoiding the use of esters of β alanine which polymerize on standing[57] and, furthermore, of yielding pantothenic acid or its calcium salt directly, thus avoiding the saponification procedure after condensation Approximately a theoretical yield can be obtained This type of condensation has also been carried out with the dry sodium salt of β alanine[54, 55, 58, 59] *and yields directly the sodium salt of pantothenic acid* The sodium salt can also be obtained[59] from the ethyl ester of pantothenic acid by hydrolysis with barium hydroxide followed by reacting the obtained barium salt with sodium sulfate Pantothenic acid has also been synthesized[60] by condensation of the lactone (III) with β alanine benzyl ester (VI) followed by catalytic hydrogenation of the pantothenic acid benzyl ester (VII) to the free pantothenic acid (V) The synthetically obtained racemic pantothenic acid can be resolved into its components by crystallization of its quinine[60, 61] quinine methohydroxide[62] or cin

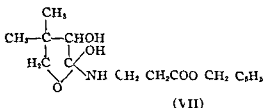
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[52] D W Woolley H A Waisman and C A Elvehjem *J Am Chem Soc* 61 977 (1939)
[53] D W Woolley H A Waisman and C A Elvehjem *J Biol Chem* 129 673 (1939)
[54] R J Williams H K Mitchell H H Weinstock and L E Snell *J Am Chem Soc* 62 1784 (1940)
[55] T Reichstein and A Grüssner *Helv Chim Acta* 23 650 (1940)
[56] R J Williams *Science* 89 486 (1939)
[57] E Abderhalden and A Fodor *Z physiol Chem* 85 118 (1913)
[58] S H Babcock and T H Jukes *J Am Chem Soc* 62 1628 (1940)
[59] M Gätzi Fichter H Reich and T Reichstein *Helv Chim Acta* 24 185 (1941)
[60] R Kuhn and T Wieland *Ber* 73 971 1134 (1940)
[61] A Grüssner M Gätzi Fichter and T Reichstein *Helv Chim Acta* 23 1276 (1940)
[62] E T Stiller and P F Wiley *J Am Chem Soc* 63 1237 (1941)

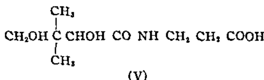
chonidine methohydroxide[61] salts The (+) compound is identical with the naturally occurring pantothenic acid





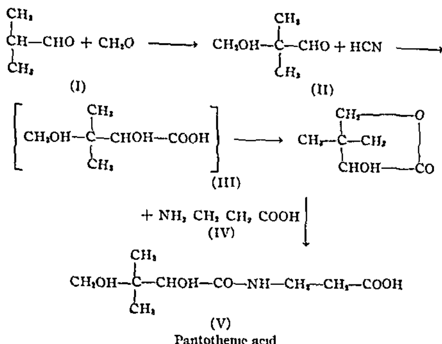






The synthesis of the α hydroxy β β dimethyl γ butyrolactone has been carried out as follows [63 64 65 66] isobutyro aldehyde (I) is condensed[67] with formaldehyde to give α α dimethyl β hydroxy propionaldehyde (II) which upon condensation with hydrocyanic acid, or better by condensation with potassium cyanide in the presence of calcium chloride[68] or by reaction of the bisulfite compound of the aldehyde (III) with potassium cyanide, yields racemic α hydroxy β,β dimethyl γ butyrolactone (III)

[63] E Glaser *Monatsh* 25 46 (1904)
[64] M Kohn and V Neustädter *Ibid* 39 293 (1918)
[65] E T Stiller S A Harris J Finkelstein J C Keresztesy and K. Folkers *J Am Chem Soc* 62 1785 (1940)
[66] T Reichstein and A Grussner *Helv Chim Acta* 23 650 (1940)
[67] L Wessely *Monatsh* 21 231 (1900)
[68] H E Carter and L F Ney *J Am Chem Soc* 63 312 (1941)



Pantothenic acid

The natural dextrorotatory pantothenic acid yields upon hydrolysis the $(+)\alpha,\gamma$ dihydroxy $\beta,\beta$ dimethyl butyric acid The lactone corresponding to this (+)dihydroxy acid is the (−) form

The racemic, synthetically obtained $\alpha$ hydroxy $\beta,\beta$ dimethyl $\gamma$ butyro lactone can be resolved by conversion into the quinine,[65] [66] quinine metho hydroxide quinidine methohydroxide or cinchonine methohydroxide[69] salt Thus, the pure $(-)\alpha\ \gamma$ dihydroxy $\beta,\beta$ dimethyl butyro lactone can be ob tained The isomeric (+) lactone can be racemized by heating the sodium salt

$\beta$ Alanine can be prepared from $\beta$ halogenated propionic acids and ammonia [70] or from succinimide by means of hypobromide and potassium hydroxide [71] Commercially $\beta$ alanine is produced by adding ammonia to the double bond of acrylic esters[7] followed by saponification of the ester with barium hydroxide An excellent alternative method is the catalytic hydrogenation of ethyl cyano acetate [3]

## 8 Industrial Methods of Preparation

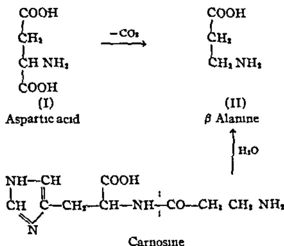
Pantothenic acid is commercially available in the form of its crystalline synthetic calcium or sodium salt prepared according to the previously

[69] R T Major and J Finkelstein J Am Chem Soc 63 1368 (1941)
W H[e]intz Ann 156 36 (1870) E Mulder B[er] 9 1903 (18[7]6)
S Hoogewerff and W A van Dorp Rec trav chim 10 4 (1891) H T Clarke and H J Behr Org Synth[es] 16 1 (1936)
[illegible] Chim Ital 19 438 (1889) K Mo[rsc]h M[onat]sh 63 220 (193[3])
[73] F W[e]yg[and] Ber 74 2[5]6 (1941)

described methods Pantothenic acid concentrates for example from rice brans, from which most of the other vitamins of the B complex are removed by treatment with fuller's earth are also marketed Extracts from certain strains of yeast and bacteria are being prepared which contain most members of the vitamin B complex including pantothenic acid The residues of liver preparations which have been worked up for the antipernicious anemia factor contain considerable amounts of pantothenic acid and are used commercially for the extraction of this vitamin

## 9 Biogenesis

The biogenesis of pantothenic acid is not known, but it is reasonable to assume that those organisms which are able to synthesize this vitamin accomplish this by condensation of β alanine with the dihydroxy dimethyl butyric acid or its lactone The biogenesis of this acid has not been elucidated β Alanine may arise from a number of different reactions Thus it has been shown that β alanine (II) is obtained by various bacteria upon decarboxylation of aspartic acid (I) [74] Certain bacteria are able to hydrolyze carnosine (III) to β alanine [75]




## 10 Specificity

Natural pantothenic acid appears to be a single substance[76] of considerable specificity Even the optical isomer, the (−) pantothenic acid is

[74] D Ackermann *Z physiol Chem* 54 1 (1907) 60 482 (1909) *Z Biol* 56 87 (1908) A I Virtanen and T Laine *Suomen Kemistilehti* B10 2 (1937) *Enzymologia* 3 266 (1937) *Nature* 142 674 (1938)

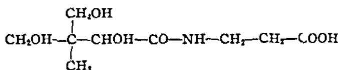
[75] J H Mueller *J Biol Chem* 123 421 (1938)

[76] H K Mitchell E E Snell and R J Williams *J Am Chem Soc* 62 1791 (1940)

inactive in bacterial[77] and rat assays [61, 78] Both the carboxyl group and the hydroxyl groups are necessary for the physiological action The acetate,[79] benzoate and diphosphate[80] of the acid are inactive [79] The salts of pantothenic acid and certain esters, such as the ethyl ester, are active [61] β Alanine alone may act as a growth stimulant for yeast [81] certain strains of diphtheria bacillus[82, 83] and to a certain extent for rats,[84, 85] but not for chicks It has been shown that organisms which depend upon an external supply of β alanine alone convert this compound into pantothenic acid [81, 83] Other microorganisms, for example lactic acid bacteria,[86] are unable to utilize only β alanine Some hemolytic bacteria have been found[87] which require only an external supply of the dihydroxy dimethyl butyric acid part of the pantothenic acid, but not the β alanine part

Chondroitin sulfuric acid has some growth promoting action on rats [85, 88] but its effect even in large doses is slight compared with that of pantothenic acid

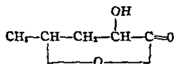
A number of compounds, similar in structure to pantothenic acid, have been prepared Of these, the most interesting one is hydroxy pantothenic acid ' (I) prepared[89] from β alanine and α hydroxy β methyl β hydroxy methyl butyrolactone This compound possesses striking biological activity which varies, according to the organism used for testing and the assay conditions, between 1 5% and 25% of pantothenic acid

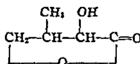


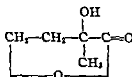

(I)
Hydroxy pantothenic acid

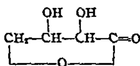
[77] E T Stiller S A Harris J Finkelstein J C Keresztesy and K Folkers *J Am Chem Soc* 62 1785 (1940)
[78] R Kuhn and T Wieland *Ber* 73 971 1134 (1940)
[79] D W Woolley H A Waisman O Mickelsen and C A Elvehjem *J Biol Chem* 125 715 (1938)
[80] D W Woolley *Ibid* 134 461 (1940)
[81] H H Weinstock H K Mitchell E F Pratt and R J Williams *J Am Chem Soc* 61 1421 (1939)
[82] J H Mueller *Proc Soc Exptl Biol Med* 36 706 (1937) J H Mueller and A W Klotz *J Am Chem Soc* 60 3086 (1938)
[83] W C Evans W R C Handley and F C Happold *Brit J Exptl Path* 20 396 (1939)
[84] M Hoffer and T Reichstein *Nature* 144 7. (1939)
[85] M M El Sadr H G Hind T F Macrae C F Work B Lythgoe and A R Todd *Ibid* 144 73 (1939)
[86] E E Snell F M Strong and W H Peterson *J Am Chem Soc* 60 2825 (1938) *Biochem J* 31 1789 (1937)
[87] D W Woolley *J Biol Chem* 130 417 (1939)
[88] H E Robinson R E Gray F F Chesley and L A Crandall *J Nutrition* 17, 2.7 (1939)
[89] H K Mitchell F E Snell and R J Williams *J Am Chem Soc* 62 1791 (1940)

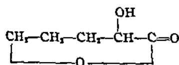
The other compounds tested possess definite, but small activity (less than a fraction of 1% of the activity of pure pantothenic acid) These compounds have been prepared by condensation of β alanine with the following acids or lactones

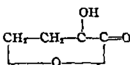

α Hydroxy γ valerolactone[90, 91]

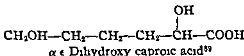

α Hydroxy β methyl γ butyrolactone[90]


α Hydroxy α methyl γ butyrolactone[90]


α β Dihydroxy γ butyrolactone[90]


α Hydroxy δ valerolactone[89, 91, 92]


α Hydroxy γ butyrolactone[90]


α ε Dihydroxy caproic acid[89]

The high structural specificity of pantothenic acid is also evident from the failure to obtain active compounds by replacing the β alanine part of the molecule with other amino acids Thus the analogous compounds obtained from α alanine, from the methyl homolog β amino butyric acid from carboxy β alanine (aspartic acid)[93] and from *l* leucine[94] proved to be inactive

## 11 Determination

There are no specific physical or chemical tests known for pantothenic acid The following biological tests have been proposed and have been used for the determination of various samples of crude preparations

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[90] H K Mitchell H H Weinstock E E Snell S R Stanbery and R. J Williams *J Am Chem. Soc* 62 1776 (1940)

[91] T Reichstein and A Grüssner *Helv Chim Acta* 23 650 (1940)

[92] Y Subbarow and L Rane *J Am Chem Soc* 61 1616 (1939)

[93] H H Weinstock E L May A Arnold and D Price *J Biol Chem.*, 135 343 (1940)

[94] R. Kuhn and T Wieland *Ber* 73 962 (1940)

1 Chick test according to Jukes and Lepkovsky [95, 96] In this test, the growth of chicks and the prevention or cure of the specific chick dermatitis is measured

2 Rat test [97, 98, 99] The increase in weight over a five week period is measured

3 Bacteria tests This is a growth test for Proteus morganii,[100, 101] lactic acid organisms[102, 103, 104, 105] and hemolytic streptococci [106] The potency of unknown material is judged by quantitative estimation of the turbidity caused by the growth of the bacteria and by titration of the acid produced during growth

4 Yeast test.[107] According to this test, the *increase in growth of* yeast following the administration of pantothenic acid is determined Using this method, five parts in ten billion parts of culture medium (0 0005 γ per ml ) can be determined quantitatively [108]

## 12 Standards

No International Unit of pantothenic acid has been established so far The sodium salt of pantothenic acid has been recommended as standard [109]

1 g pantothenic acid = 71 000 Chick Units [110]
= 50 000 000 Streptobacterium Units [111]

Yeast Unit One Yeast Unit[108] of pantothenic acid is that amount which when tested by the yeast growth method[107, 108] is equivalent to one g of a standard dry rice bran extract This extract is prepared from rice bran with 60% methanol

Ratio Yeast Unit to Chick Unit = 5 1 [112]

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[95] T Jukes and S Lepkovsky *J Biol Chem* 111 119 (1935) 114 109 117 (1936)
[96] T H Jukes *Ibid* 117 11 (1937)
[97] C E Edgar M M El-Sadr and T F Macrae *Biochem J* 32 2200 (1938)
[98] C E Edgar and T F Macrae *Ibid* 31 886 893 (1937)
[99] G H Hitchings and Y Subbarow *J Nutrition* 18 265 (1939)
[100] M J Pelczar and J R Porter *Proc Exptl Biol Med* 43 151 (1940)
[101] M J Pelczar and J R Porter *J Biol Chem* 139 111 (1941)
[102] E E Snell D Pennington and R J Williams *Proc Am Soc Biol Chem J Biol Chem* 133 XCII (1940)
[103] E E Snell F M Strong and W H Peterson *Biochem J* 31 1789 (1937) *J Bact* 38 293 (1939)
[104] D Pennington E E Snell and R J Williams *J Biol Chem* 135 213 (1940)
[105] E E Snell and L D Wright *Proc Am Soc Biol Chem* 1941 CXIX
[106] Y Subbarow and L Rane *J Am Chem Soc* 61 1616 (1939)
[107] R J Williams and D H Saunders *Biochem J* 28 1887 (1934) R J Williams E D McAlister and R R Roehm *J Biol Chem* 83 315 (1929)
[108] R J Williams J H Truesdail H H Weinstock E Rohrmann C M Lyman and C H McBurney *J Am Chem Soc* 60 2719 (1938)
[109] M Gätzi Fichter H Reich and T Reichstein *Helv Chim Acta* 24 185 (1941)
[110] L W McElroy and H Goss *J Biol Chem* 130 437 (1939)
[111] R Kuhn and T Wieland *Ber* 73 971 1134 (1940)
[112] T H Jukes *J Am Chem Soc* 61 975 (1939)

**Chick Unit** One Chick Unit[113] of pantothenic acid is defined as one tenth of the amount which will just provide for maximal growth when fed daily to a chick three weeks old in conjugation with a diet free of this vitamin

1 Chick Unit = 14 γ pantothenic acid [110]

**Sbm Unit (Streptobacterium Unit)** One Sbm Unit of pantothenic acid has been defined as that amount of the acid which under standardized conditions must be present in one cc of a culture medium in order to achieve maximum cell growth of *Streptobacterium plantarum* [114]

## 13 Physiology of Plants and Microorganisms

Plants and microorganisms may be classified with respect to pantothenic acid into those which synthesize this vitamin and those which need an external supply [115] The latter group may be subclassified into those which need the entire pantothenic acid molecule for example lactic acid [116] and propionic acid [117] bacteria streptococci and pneumococci,[118] those which need only the β alanine part and those which need only the aliphatic di hydroxy carboxylic acid part (for examples see page 264)

The fact that pantothenic acid plays a role in the growth of plants and microorganisms is undeniable but the extent of that role is undetermined At the present time only a stimulating action of pantothenic acid has been proved for example for pea embryos [119] The actual necessity of this compound for plant life especially green plant life, has not been con vincingly demonstrated [120]

Green plants synthesize pantothenic acid after they develop their photo-synthetic apparatus Yeast synthesizes small amounts but only in the absence of an external supply [121,122] that is yeast is a parasitoid On the other hand, yeast synthesizes pantothenic acid *ad libitum* from administered β alanine [121]

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[113] T H Jukes *J Biol Chem* 117 11 (1937)
[114] R Kuhn and T Wieland *Ibid* 73 962 (1940)
[115] E J Krauskopf E E Snell and E McCoy *Enzymologia* 7 327 (1939)
[116] E E Snell F M Strong and W H Peterson *J Am Chem Soc* 60 2825 (1938) *J Bact* 38, 293 (1939)
[117] E J Krauskopf E E Snell and E McCoy *Enzymologia* 7 327 (1939)
[118] L Rane and Y Subbarow *J Biol Chem* 134 455 (1940)
[119] J Bonner and G Axtman *Proc Natl Acad Sci U S* 23 453 (1937)
[120] R J Williams and E Rohrman *Plant Physiol* 10 559 (1935)
[121] H H Weinstock H K Mitchell E F Pratt and R J Williams *J Am Chem Soc* 61 1421 (1939)
[122] R J Williams W A Mosher and E Rohrmann *Biochem J* 30 2036 (1936)

## 14 Animal Physiology

Very little is known about the physiology of pantothenic acid and its metabolism in man and animals Since pantothenic acid is a vitamin it must be concluded that it is adsorbed from the intestinal tract but it is not known if it is adsorbed as such or after partial or total hydrolysis into the two chemically different parts It may be assumed that in the intestines pantothenic acid is freed from the protein material to which it is bound in animal tissues although some evidence exists that the bound vitamin is considerably less well utilized than the free vitamin Blood contains a constant amount of this vitamin and the liver and kidneys are apparently able to store this compound to a certain extent In tissues from chicks fed a pantothenic acid deficient diet the content of this vitamin was found decidedly lower than normal [123] In the spinal cord, brain muscle and blood the differences exceeded 50%, in the liver and kidneys 65% [124] The blood of men having symptoms of deficiencies of the vitamins $B_1$ $B_2$ or nicotinic acid showed also a decrease of 25–50% of the normal level of pantothenic acid [125] In man, the pantothenic acid content in the serum is increased after injection, but returns to the normal level within one day [126] Pantothenic acid is partly destroyed in the organism and is constantly excreted through the urine [127]

In weanling rats kept on a diet deficient in pantothenic acid, the amount of liver fat is markedly less than in control animals kept on a non-deficient diet Addition of pantothenic acid increases the liver fat [128] The low levels of liver fat can, however, to a certain extent be related to low food intake during the vitamin deficiency

## 15 Avitaminosis and Hypovitaminosis

The symptoms of a pantothenic acid deficiency in man are largely unknown and the human need for this vitamin has not been demonstrated However, since this compound is necessary for rats and chicks, it seems reasonable to assume that it will prove of necessity to man Indeed a lowering of the pantothenic acid level in blood has been observed in pellagra, beriberi and riboflavin deficiency [129]

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[123] E E Snell D Pennington and R J Williams *J Biol Chem* 133 559 (1940)
[124] E E Snell D Pennington and R J Williams *Proc Am Soc Biol Chem* 34 XCII (1940)
[125] S R Stanbery E E Snell and T D Spies *J Biol Chem* 135 353 (1940)
[126] T D Spies S R Stanbery R J Williams T H Jukes and S H Babcock *J Am Med Assoc* 115 523 (1940)
[127] D Pennington E E Snell and R J Williams *J Biol Chem* 135 213 (1940)
[128] R W Engel *Proc Am Soc Biol Chem* 1941 XXXVII
[129] S R Stanbery E E Snell and T D Spies *J Biol Chem* 135 353 (1940)

Chicks on a pantothenic acid deficient diet suffer from a specific dermatosis Incrustations occur about the eyes the corners of the mouth and the areas between the toes [130] The skin epithelium becomes keratinized and a dry sloughing sets in Feathering is retarded and the feathers produced are rough Besides the dermatitis certain lesions occur in the spinal cord, characterized by degeneration of the myelinated fibers [131] Chicks suffering from the specific dermatitis often show thymus involution and liver damage (fatty livers) [131]

Domestic fowls need pantothenic acid for hatchability and reproduction [132] not, however for egg production [133] The rate of hatching eggs has been shown to drop from 70% to 3% when the vitamin was removed from the hen s diet

Pantothenic acid deficiency in young rats[134, 135] and mice[136] causes retardation of growth A specific symmetrical depigmentation of the fur (nutritional achromotrichia) sets in in rats and mice[137, 138, 139, 140, 141] which has also been observed in foxes especially silver foxes As the result of this deficiency the fur turns gray [139] In albino rats a rustiness of the fur develops[142] and blood caked whiskers have been observed regularly [140]

In rats on a pantothenic acid deficient diet a marked adrenal hemorrhage, atrophy and necrosis have been observed [143, 144, 145] No such adrenal changes were found in mice [13] Hemorrhages under the skin have also been observed in rats [140]

Another symptom in rats which might be cured by pantothenic acid is the so called 'spectacled eye condition [146, 147] (See pages 280 and 477 )

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[130] L C Norris and A T Ringro e *Science* 71 643 (1930) A F Ringrose L C Norr s and G I Heus r *Poult y Sci* 10 166 (1931)

[131] P H Phillips and R W Engel *J Nut i on* 18 2?7 (1939)

[132] T H Jukes *J Biol Chem* 129 2? (1939)

[133] J C Bauernf nd and L C Norris *Science* 89 41( (1939)

[134] C E Edgar M M El S d and T F Macrie *B ochem J* 32 00 (1938)

[135] C H Hit h ngs and Y Subbarow *J Nut iti n* 18 ?65 (1939)

[136] H P Morris and S W L pp n ott *P oc Am S c B ol Chem* 1941 XCIII

[137] P György C E Poling and Y Subbarow *J Biol Chem* 132 789 (1940)

[138] P György and C E Poling *Science* 92 20_ (1940)

[139] P György and C E Pol ng *Proc Soc Exptl Biol Med* 45 773 (1940) K Unna G V Richards and W I Sampso *J N tri on* 22 553 (1941)

[140] K. Unna *Proc Am Physiol Soc* 1941 285 *J Nut tion* 20 565 (1940)

[141] M K. D mick and A Lepp *J Nut ition* 20 413 (1940)

[142] H S Owens M Trautman and E Woods *Scie ce* 93 406 (1941)

[143] F S Daft and W H Sebrell *U S Pub Health Service Pub Health Repts* 54 2217 (1939)

[144] F S Daft W H Sebrell S H Babcock and T H Jukes *Ib d* 55 1333 (1940)

[145] L L Ashburn *Ib d* 55 1337 (1940)

[146] J J Oleson H R B rd C A Elvehjem and E B Hart *J B ol Chem* 127 23 (1939)

[147] J Goldberger and R D Lill e *U S Pub Health Serv ce Pub Health Repts* 41 10 5 (19 6) A Bourqu n and H C Sherman *J Am Chem Soc* 53 3501 (1931) H E Robinson and R C Newton Abstracts D isions of B ological Chem try Am Ch m Soc Kansas City April 13-17 (1936) S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 557 (1936)

It has also been postulated that pantothenic acid deficiency causes an earlier aging

## (a) *Clinical Test Methods*

In man and in animals, pantothenic acid can be determined by bacteriological methods either in blood or in urine For *blood determinations*[148] fresh venous blood is citrated to prevent clotting The diluted material is then tested according to the bacteria tests as previously described In normal human beings, the pantothenic acid content of blood ranges from 0 19 to 0 32 $\gamma$ per cc and an average of 0 225 $\gamma$ per cc has been noted In patients with vitamin B-complex deficiency a lowered value of 0 05 to 0 09 $\gamma$ per cc. has been observed In *urine determinations,*[149] which are best *carried out according to the bacteria tests,* it is advisable to run check determinations with samples in which the pantothenic acid has been destroyed chemically or to which a known amount of pantothenic acid has been added

## 16 Hypervitaminosis

Pantothenic acid is essentially non toxic At least 100 mg may be injected *intravenously into* man *without producing any toxic reactions*[150]

## 17 Requirements

Pantothenic acid is probably required by all living matter It has been shown to stimulate the growth of bacteria (lactic acid bacteria,[151] pneumococcus,[152] propionic acid bacteria[153] streptococci,[153] diphtheria bacillus,[154] molds (*aspergillus niger*)), protozoa, fungi, seed plants (alfalfa[155]), higher animals (mice, rats[156] [157] fox pigs, dogs[158] birds[159] [160]) and is

[148] S R Stanbery E F Snell and T D Spies *J Biol Chem* 135 353 (1940)

[149] D Pennington E E Snell and R J Williams *Ibid* 135 213 (1940)

[150] K Unna and J Greslin *Proc Soc Exptl Biol Med* 45 311 (1940) T D Spies D P Hightower and L H Hubbard *J Am Med Assoc* 115 292 (1940)

[151] S Orla Jensen N C Otte and A Snog Kjaer *Kgl Danske Videnskab Selskab Skrifter Naturvidenskab math Afdel* 6 No 5 52 pp (1936) *Zentr Bakt Parasitenk* (II) 94 434 (1936)

[152] L Rane and Y Subbarow *J Biol Chem* 134 455 (1940)

[153] E J Krauskopf E E Snell and E McCoy *Enzymologia* 7, 327 (1939)

[154] Y Subbarow and L Rane *J Am Chem Soc* 61 1616 (1939)

[155] C H McBurney W B Bollen and R. J Williams *Proc Natl Acad Sci U S* 21 301 (1935)

[156] S Lepkovsky T H Jukes and M E Krause *J Biol Chem* 115 557 (1936)

[157] Y Subbarow and G H Hitchings *J Am Chem Soc* 61 1615 (1939)

[158] O Mickelsen H A Waisman and C A. Elvehjem *J Biol Chem* 124 313 (1938) D W Woolley H A Waisman O Mickelsen and C A Elvehjem *Ibid* 125 715 (1938) J M. McKibbin S Black and C A Elvehjem *Am J Physiol* 130 365 (1940)

[159] J C Bauernfeind and L C Norris *Science* 89 416 (1939)

[160] T H Jukes *J Biol Chem* 129 225 (1939)

probably essential also for human beings While sheep[161] and cattle[162, 163, 164] require pantothenic acid, they do not need an external supply since it is synthesized by microorganisms in the rumen

The amounts required by the various organisms are largely unknown Yeast needs as growth stimulant 0 008 $\gamma$ of a purified pantothenic acid per cc of culture medium In rats a single dose of 800 $\gamma$ given to depleted animals produced a rapid and marked gain in weight [165] The optimal daily dose for rats is above 10 $\gamma$[166] and about 100 $\gamma$ are needed for the cure or prevention of achromotrichia[167] and hemorrhagic adrenal necrosis [168, 169] For chicks about 500 $\gamma$ per 100 g of diet appear to be necessary to prevent the typical dermatitis and about 600 $\gamma$ to insure optimal growth

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161 L W McElroy and H Goss *J Biol Chem* 130 437 (1939)

162 L W McElroy and H Goss *Ibid* 133 LXV (1940)

163 M I Wegner A N Booth C A Elvehjem and E B Hart *P oc Soc Exptl Biol Med* 45 769 (1940)

164 L W McElroy and H Goss *J Nutrit on* 21 405 (1941)

165 E T Stiller S A Harr s J Finkelstein J C Keresztesy and K Folkers *J Am Chem Soc* 62 1785 (1940)

166 A Grussner M Gätzi Fichter and T Reichstein *Helv Chim Acta* 23 1276 (1940)

167 P György and C E Pol ng *Science* 92 20? (1940)

168 F S Daft W H Sebrell S H Babcock and T H Jukes *U S Pub Health Service Pub Health Repts* 55 1333 (1940)

169 L L Ashburn *Ibid* 55 1337 (1940)

# INOSITOL

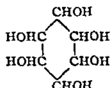
# INOSITOL

## 1 Nomenclature

**Names**

Inositol or more accurately meso or inactive inositol (i inositol)
Inosite
Cyclohexanehexol
Bios I
Mouse antialopecia factor [1]
*Rat anti spectacled eye factor* [2] (*See also pages* 269 *and* 477)

**Chemical formula**




**Empirical formula**

$C_6H_{12}O_6$

## 2 Chronology

1901 WILDIERS[3] recognized that yeast needs for optimal growth a special material bios in addition to the known nutrients This had previously been postulated by LIEBIG in 1869

1924 LUCAS[4] separated bios into two components bios I and II

1928 EASTCOTT[5] isolated bios I in the pure form and identified it with the long known inactive inositol

1940 WOOLLEY[6] produced an alimentary deficiency alopecia in mice and identified the missing factor with inositol

## 3 Occurrence

Inositol occurs as a normal cell constituent in practically all plant and animal tissues In plants the highest amounts are found in the leaves

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[1] D W Woolley *J Biol Chem* 136 113 (1940)
[2] P L Pavcek and H M Baum *Science* 93 502 (1941)
[3] E Wildiers *La cellule* 18 313 (1901)
[4] G H W Lucas *J Phys Chem* 28 1180 (1924)
[5] E V Eastcott *Ibid* 32 1094 (1928)
[6] D W Woolley *Science* 92 384 (1940)

The concentration varies according to the season and reaches its maximum shortly before the time that the fruit ripens Fruits, especially citrus fruit (lemon, orange, grapefruit), are good sources of inositol [7] Cereal grains are also rich sources Yeast molds and bacteria, at least certain species contain large amounts of inositol [8] Inositol occurs in the animal organism both in the tissues and in the body fluids Thus inositol has been isolated from skeletal and heart muscle lungs kidneys, liver brain blood, milk, urine eggs, etc Inositol is also found in the eye lens and the optical nerve [9]

Inositol occurs naturally in a number of different forms In liver it exists in an alkali labile combination with a large molecule, probably a protein A combined form also exists in the heart muscle[10, 11] (for example, that of dogs) and perhaps other types of muscles Cardiac muscles of sheep, pigs and oxen[12] contain if any, only very small amounts in the combined form Some inositol appears to occur in the free state In plants the majority is present in the form of the hexa phosphate (phytic acid) In the tubercle bacillus[13] and the soybean inositol is bound in the phosphatide fraction as a glucoside [14]

## 4 Isolation

*Inositol is obtained, for example, from muscles, liver or leaves by hydrolysis* with aqueous potassium hydroxide or calcium hydroxide or with concentrated hydrochloric acid,[15] followed by precipitation of large amounts of by products with normal lead acetate The inositol is then precipitated with basic lead acetate and, after removal of the lead is precipitated by baryta in alcoholic solution The free inositol is obtained from the barium precipitate by means of carbon dioxide and is recrystallized for example, from a minimum amount of water to which alcohol and ether are added to complete the crystallization [16] *Inositol may be prepared from phytin* by hydrolysis with formic acid[17] or with calcium hydroxide

*i* Inositol can be identified as the hexa acetate m p 210° C

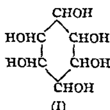
[7] E K Nelson and G L Keenan *Science* 77 561 (1933)
[8] F Kögl and W van Hasselt *Z physiol Chem* 242 43 (1936)
[9] A C Krause and R Weekers *Arch Ophthalmol* 20 299 (1938)
[10] L B Winter *Biochem J* 28 6 (1934)
[11] F Rosenberger *Z physiol Chem* 64 341 (1910)
[12] L B Winter *Biochem J* 34 249 (1940)
[13] R J Anderson and E G Roberts *J Biol Chem* 89 599 611 (1930)
[14] R J Anderson W C Lothrop and M M Creighton *Ibid* 125 299 (1938)
[15] D W Woolley *Ibid* 139 29 (1941)
[16] Maquenne *Compt rend* 104 225 (1886) *Ann chim phys* [6] 12 89 (1887) A. Cloetta *Ann* 99 289 (1856)
[17] K Lindenfeld *Biochem Z* 272 284 (1934)

## 5 Properties

Inositol has a sweet taste The dihydrate melts at 215–216° C while the anhydrous compound melts at 225–226° C Inositol is soluble in water (one part in 5 7 parts of water at 24° C ), but is insoluble in absolute alcohol and in ether It crystallizes from acetic acid or from water above 50° C as the water free compound but below 50° C as the dihydrate

## 6 Chemistry

Inositol has the formula of a hexahydroxy cyclohexane (I) Theoretically, the latter can exist in nine different cis trans isomers



(I)


(II)

Inositol is optically inactive and is often called meso inositol It has the configuration (II) and forms various esters such as a hexa acetate a hexa phosphate etc

## 7 Industrial Methods of Preparation

Inositol is not a commercially important product However due to the abundant occurrence of this compound in all sorts of natural materials it should not be difficult to find a cheap raw material which contains relatively high amounts The recovery of inositol from the steep water of starch factories for example from corn starch plants is of potential commercial value [18] Inositol can also be prepared synthetically by hydrogenation of hexa hydroxy benzene [19]

## 8 Biogenesis

The mechanism of the inositol formation in plants is not definitely known, but it is generally assumed that inositol is synthesized by aldol type condensations from carbon dioxide and water under the influence of light [9] or

[18] E Bartow W W Walker and F A. Hoglan *Am Y cong intern chem* 4 361 (1939)
[19] H Wieland and R. S Wishart *Ber* 47 2082 (1914)
[20] R. Kögel *Biochem Z* 95 313 (1919) 97 21 (1919)

from hexoses by ring closure [21] Inositol is apparently also synthesized by the chick embryo After about the seventh day of incubation the inositol concentration starts to increase and eventually becomes six times that present in the original egg [22]

## 9 Specificity

Little is known about the specificity of inositol The optical isomers of *i inositol are inactive* The compounds reported to be active in mice are, besides inositol, the naturally occurring phosphoric acid ester, tested as the calcium magnesium salt phytin,[23] inositol hexa acetate and soybean cepha lin [24] Mytilitol (methyl inositol) is also active Only inositol, and not its esters, is active as a growth factor for yeast [24]

## 10 Determination

Specific methods have not been worked out for the determination of inositol especially in natural materials A *chemical method* based on an oxidation with potassium iodo mercurate[25 26] can be used for determination of about 1–5 mg but is not specific since many polyhydroxy compounds in cluding glycol, mannitol, etc, can be oxidized similarly A *biological method* based on the ability of inositol to cure or prevent alopecia in mice has occasionally been used [27 28 29] The best method is undoubtedly the *microbiological method* in which the growth of, for example, yeast is meas ured as a function of the amount of inositol present This procedure has, however, not been perfected

## *11 Physiology of Plants and Microorganisms*

A number of different strains of yeast have been investigated for the growth promoting effect of inositol Some require an external supply of inositol for optimum growth while others show no beneficial effect upon the addition of inositol to the culture medium [30 31] Similarly, some fung

[21] F Micheel H Ruhkopf and F Suckfull *Ber* 68 1523 (1935)
[22] E E Snell and E Quarles *J Nutrition* 22 483 (1941)
[23] D W Woolley *Science* 92 384 (1940)
[24] D W Woolley *J Nutrition* 21 Supplement 17 (1941)
[25] L Young *Biochem J* 28 1428 1435 (1934)
[26] R A Gregory *Ibid* 29 2798 (1935)
[27] D W Woolley *J Biol Chem* 136 113 (1940)
[28] D W Woolley *Ibid* 139 29 (1941)
[29] D W Woolley *J Nutrition* 21 Supplement 17 (1941)
[30] H Stantial *Trans Roy Soc Can* III 26 163 (1932)
[31] J B Lesh L A. Underkofler and E I Fulmer *J Am Chem Soc* 60 2505 (1938)

require inositol while others are apparently able to synthesize this compound[32]

In higher plants for example, in the tobacco plant, the inositol content increases during growth until maturity is reached Thereafter the inositol content decreases slowly Tobacco seeds contain little inositol[33] Some plants or microorganisms apparently excrete considerable amounts of inositol into the soil since this compound has been isolated in appreciable quantities from agricultural soils[34]

There is also the possibility that inositol is used, at least by some species, not only as a growth stimulant but also as a building unit Thus in caoutchouc a mono and a dimethyl ether of inositol have been found, the physiological significance of which is not known

## 12 Animal Physiology

While little is known about the physiological action of inositol, a few pertinent observations have been made Inositol occurs in animal tissues to a certain extent in the free form, but also as the phosphoric acid ester for example in the blood of chicken[35] It is thus possible that inositol is phosphorylated in the organism The possibility however cannot be excluded that the naturally occurring phosphate is directly absorbed

The mechanism of the inositol action is not known It has been shown that certain bacteria oxidize one of the hydroxyl groups of inositol to a keto group, but it is not known whether or not such an oxidation occurs in the animal organism The physiological significance of this oxidation has not been elucidated It has been claimed that inositol can be oxidized to compounds of unknown composition in muscle liver, kidney and brain, but in other laboratories no significant effect upon the respiration of the brain tissue of the rat or the rabbit could be observed[36]

Inositol markedly increases the peristalsis of the stomach and the small intestine[37] It has been suggested that inositol is the nutritional factor which determines gastrointestinal motility[37]

Inositol appears to act as a lipotropic factor In rats fed a synthetic diet supplemented with the vitamins $B_1$ $B_2$ $B_6$ pantothenic acid choline and biotin fatty livers containing large amounts of cholesterol are pro

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[32] F Kögl and N Fries *Z phys ol Chem* 249 93 (1937)
[33] A P Smirnov *State S R Inst Tobacco Makho ka Ind (U S S R.)* No 140 115 (1939)
[34] R K Yosh da *Soil Sci* 50 81 (1940)
[35] S Rapopert *J B ol Chem* 135 403 (1940)
[36] B C Guha and N Das *Current Sci* 3 157 (1934) *Z physiol Chem* 231 157 (1935) L Young *P oc Soc Expll B ol Med* 35 507 (1936)
[37] G J Martin M R Thompson and J de Carvajal Forero *Am J Digestive Dis* 8 290 (1941)

duced The development of this special type of fatty liver is prevented by feeding inositol[38]

## 13 Avitaminosis

Inositol deficiency symptoms have been noted, especially in young mice[39, 40] and in rats[41] These rodents require inositol for normal growth and maintenance of hair White mice on an inositol deficient diet become completely hairless over the trunk and severe dermatitis follows In rats, the denudation sets in around the eyes and results in the so called spectacled eye syndrome[42] Inositol deficiency in rats causes also the development of a special type of fatty liver containing large amounts of cholesterol[43]

## 14 Hypervitaminosis

No ill effects have been observed from an intake of inositol but the true toxicity threshold of inositol has not been determined

## 15 Requirements

The optimum requirements of inositol are largely unknown Deficiency symptoms have been cured in white mice by feeding 10 mg of inositol per 100 g of food[40] and in rats by feeding a daily dose of 20 mg[41] The requirements of other animals are not known The human requirements have also not been established

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[38] G Gavin and E W McHenry *J Biol Chem* 139 485 (1941)
[39] D W Woolley *Ibid* 136 113 (1940) *Science* 92 384 (1940)
[40] G J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)
[41] P L Pavcek and H M Baum *Am Chem Soc St Louis Meeting* April 1941 Div Biol Chem Abstr p 2
[42] P L Pavcek and H M Baum *Science* 93 502 (1941) (See however pages 269 and 477 )
[43] G Gavin and E W McHenry *J Biol Chem* 139 485 (1941)

# PARA-AMINO-
# BENZOIC ACID

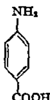
# PARA-AMINO-BENZOIC ACID

## 1 Nomenclature and Survey

**Names**

*p*-Amino-benzoic acid
Vitamin $B_x$[1a]
$B_x$ Factor[1]
Chromotrichia factor[2]
Anti gray hair factor
Anticanitic vitamin.[2]
Trichochromogenic factor[2b]
Growth factor P for bacteria[3]

**Chemical formula**




**Empirical formula**

$C_7H_7O_2N$

## 2 Chronology

1940 WOODS and FILDES[4] observed in *in vitro* experiments an anti sulfanilamide action of *p* amino benzoic acid WOODS[5] found that yeast extracts contain a factor which counteracts the sulfanilamide activity and suggested that this factor is identical with *p*-amino-benzoic acid SELBIE[6] was able to inhibit by means of *p*-amino-benzoic acid the action of sulfanilamide in mice infected with *Streptococcus haemolyticus* RUBBO and GILLESPIE[7] isolated *p*-amino-benzoic acid from yeast FILDES[8] concluded that *p*-amino-benzoic acid is an essential metabolite for bacteria

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[1] B Sure *Proc Am Soc. Biol Chem* 1941 CXXX *Science* 94, 167 (1941)
[1a] G Lunde and H Kringstad N sk *Pelsdyrblad* 13 500 (1939)
[2] S. Ansbacher *Science* 93 164 (1941)
[2a] L. L. Lachat, *Science* 93 452 (1941) V H. Kugel *Am J Med Sci.* 20? 629 (1941)
[2b] A. L. Bacharach *Food* 10 219 (1941)
[3] H N Green *Brit J Exptl Path* 21 38 (1940)
[4] D D Wood and P Fildes *J Soc. Chem Ind* 59 133 (1940)
[5] D D Woods *Brit J Exptl Path.*, 21 74 (1940)
[6] F R. Selbie *Ibid* 21 90 (1940)
[7] S. D Rubbo and J M Gillespie *Nature* 146, 838 (1940)
[8] P Fildes, *Lancet* 238, 955 (1940)

1941 ANSBACHER[9] found that *p* amino benzoic acid is a vitamin namely a chromotrichia factor for the rat and a growth promoting factor for the chick SIEVE[10] demonstrated that nutritional achromotrichia in man is favorably influenced by *p* amino benzoic acid

## 3 Occurrence, Isolation and Properties

*p* Amino benzoic acid is apparently widely distributed[10a] over the entire plant and animal kingdom but no quantitative data are available *p* Amino benzoic acid is a natural constituent of yeast and has been isolated therefrom as the benzoyl derivative [11] *p* Amino benzoic acid occurs both in a free and combined form [11a]

*p*-Amino benzoic acid is a colorless substance which melts at 186–187° C

## 4 Determination

A number of chemical and biological methods have been used or suggested for the detection of *p* amino benzoic acid None of these, however, has been sufficiently investigated to determine its degree of specificity

### (a) Chemical Method

*p* Amino benzoic acid in glacial acetic acid yields upon addition of *p* dimethyl amino benzaldehyde in glacial acetic acid a deep yellow color, which can be measured colorimetrically [12] It has been stated that this method is quite specific and only the *o* and *m* isomers and their derivatives, and aniline and its derivatives, give this test The other vitamins aliphatic amino acids and their aromatic derivatives do not respond to this reagent

### (b) Biological Methods

Biological methods for the determination of *p* amino benzoic acid have not been worked out very well Principally, either the cure or prevention of achromotrichia in rats[13] or the growth of chickens[13] can be used as a criterion for biological determinations A more simple method would be a microbiological test which makes use of the inhibitory action of *p* amino benzoic acid toward the bactericidal effect of sulfonamides It would seem possible also to develop an assay procedure employing a

[9] S Ansbacher *Science* 93 164 (1941)
[10] B F Sieve *Ibid* 94 257 (1941)
[10a] N S Dimond *Science* 94 420 (1941) S Wiedling *Science* 94 389 (1941)
[11] S D Rubbo and J M Gillespie *Nature* 146 838 (1940)
[11a] K C Blanchard *J Biol Chem* 140 919 (1941)
[12] H Tauber and S Laufer *J Am Chem Soc* 63 1488 (1941)
[13] S Ansbacher *Science* 93 164 (1941)

bacterium which needs *p* amino benzoic acid for growth such as *Brucella abortus*[14] or *Streptococcus haemolyticus*[15] Besides growth the production of acid could probably be used as a measure of the *p* amino benzoic acid present when organisms like *Clostridium acetobutylicum*[16] are used as test objects

## 5 Physiology

Little is known about the physiological action of *p* amino benzoic acid Excess amounts given either orally or parenterally are converted in the human and animal organism[17] into the acetate which is secreted through the urine

In the organism *p* amino benzoic acid counteracts the action of hydroquinone[18] which for example, in the cat[19] and in mice[20] causes a graying of the fur The action of sulfanilamide[21,22] for example in mice infected with *Streptococcus haemolyticus*[23] and the action of sulfapyridine in mice infected with *Pneumococcus*[24] are also inhibited by *p* amino benzoic acid

In vitro para amino benzoic acid modifies melanin formation[24a] and its possible role in pigmentation processes was suggested[24b] It has a ronounced influence on tyrosinase activity,[24c] inhibits the oxidative destruction of adrenaline[24d] and appears to act by blocking enzymes[24e] Experimental evidence for Woods hypothesis[24f] that para amino-benzoic acid and sulfonamides have a common point of attack on some enzyme system or systems is important[24g] The sulfonamide inhibition as influenced by para amino benzoic acid was mathematically analyzed[24h] and found to be of the competitive type as was already experimentally demonstrated[24i]

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[14] H N Green *Brit J Expll Path* 21 38 (1940)

[15] F R Selbie *Ibid* 21 90 (1940)

[16] S D Rubbo and J M Gillespie *Nature* 146 8 8 (1940)

[17] A Ellinger and M Hensel *Z physiol Chem* 91 37 (1914) B Harrow F W Power and C P Sherwin *Proc Soc Expll Biol Med* 24 422 (1926–27) K Bernhard *Z physiol Chem* 267 91 (1940)

[18] G J Martin and S. Ansbacher *J Biol Chem* 138 441 (1941)

[19] H Oettel *Arch expll Path Pharmakol* 183 319 (1936)

[20] G J Martin *Proc Am Assoc Advance Sci Pharm Section* January 1941

[21] D D Woods and P Fildes *J Soc Chem Ind* 59 133 (1940)

[22] M Landy and J Wyeno *Proc Soc Expll Biol Med* 46 59 (1941)

[23] F R Selbie *Brit J Expll Path* 21 90 (1940)

[24] M McCarty *Proc Soc Expll Biol Med* 46 133 (1941)

[24a] G J Martin W A Wisansky and S Ansbacher *Proc Soc Expll Biol Med* 47 26 (1941)

[24b] F Lipmann *J Biol Chem* 139 977 (1941)

[24c] W A Wisansky G J Martin and S Ansbacher *J Am Chem Soc* 63 1771 (1941)

[24d] W A Wisansky G J Martin C T Tchiowski and S Ansbacher *J Am Chem Soc* 64 in press (1942)

[24e] G J Martin C T Tchiowski W A Wisansky and S Ansbacher *Am J Physiol* 127 in press (1942)

[24f] D D Woods *Brit J Expll Path* 21 74 (1940)

[24g] J Klimmig *Klin Wochsch* 20 204 (1941)

[24h] O Wyss *Proc Soc Expll Biol Med* 48 122 (1941)

[24i] W A Wisansky G J Martin and S Ansbacher *J Am Chem Soc* 63 1771 (1941)

## 6 Avitaminosis

A deficiency in the *p* amino benzoic acid intake causes a graying of the fur in the black or piebald rat, a syndrome which is called nutritional achromotrichia [25] A retardation of growth has been observed in chicks on a *p* amino benzoic acid deficient diet [25] Nutritional achromotrichia in man has also been cured with *p* amino benzoic acid [26] In the female albino rat also a disturbance in the lactation has been noted [27] *p* Amino benzoic acid seems to have a therapeutic effect in certain types of asthma,[27a] possibly because of its protecting or sparing action on adrenaline [27b]

## 7 Hypervitaminosis

*p*-Amino benzoic acid is essentially non toxic [28]

## 8 Requirements

The requirements of *p* amino benzoic acid by various species have not been determined In rats 0 75 mg of the acid per day cures nutritional or hydroquinone achromotrichia [29 30] *In mice, 0 25 mg daily prevents de* pigmentation of the fur [30a] Chicks have been given[29] 30 mg per 100 g of ration, but the lowest optimum amount has not been established In human therapy a dose of 100 mg twice daily has been used successfully [31]

*p* Amino benzoic acid is required as a growth factor by many bacteria[32] such as *Clostridium acetobutylicum*,[33] *Brucella abortus*[34] and *Streptococcus haemolyticus*,[35] and the addition of this vitamin to all routine culture media has been recommended [36]

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[25] S Ansbacher *Science* 93 164 (1941)
[26] B F Sieve *Ibid* 94 257 (1941)
[27] B Sure *Proc Am Soc Biol Chem* 1941 CXXX *Science* 94 167 (1941)
[27a] G J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)
[27b] W A Wisansky G J Martin C T Tchiowski and S Ansbacher *J Am Chem Soc* 64 in press (1942)
[28] E Strauss F C Lowell and M Finland *J Clin Investigation* 20 189 (1941)
[29] S Ansbacher *Science* 93 164 (1941) S Ansbacher and M Landy *Proc Soc Exptl Biol Med* 48 3 (1941) R Richardson and A G Hogan *Proc Soc Exptl Biol Med* 48 459 (1941)
[30] G J Martin and S Ansbacher *J Biol Chem* 138 441 (1941)
[30a] G J Martin and S Ansbacher *Proc Soc Exptl Biol Med* 48 118 (1941)
[31] B F Sieve *Science* 94 257 (1941)
[32] P Fildes *Lancet* 238 955 (1940)
[33] S D Rubbo and J M Gillespie *Nature* 146 838 (1940) J O Lampen and W H Peterson *J Am Chem Soc* 63 2783 (1941)
[34] H N Green *Brit J Exptl Path* 21 38 (1940)
[35] F R Selbie *Ibid* 21 90 (1940)
[36] C A Janeway *J Am Med Assoc* 116 941 (1941)

# VITAMIN C—
# ASCORBIC ACID

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# VITAMIN C—ASCORBIC ACID

## 1 Nomenclature and Survey

**Names**

Vitamin C [1]
Ascorbic acid [2]
Cevitamic acid [3]

**Historical names**

Hexuronic acid [4]
Antiskorbutin (Holst 1912)
Antiscorbutic vitamin
Scorbutamin [5]

**Chemical names**

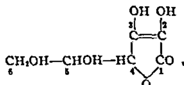
*l* Ascorbic acid
*l* Threo-3 keto-hexuronic acid lactone
*l* Xylo-ascorbic acid
3 Keto *l* gulo-furano-lactone
*l*,3 Keto-threo-hexuronic acid lactone
*l*-Threo-2 3 4 5 6 pentoxy hexen 2 carboxylic acid lactone

For the purpose of convenience a system of nomenclature has been adopted for compounds related to ascorbic acid in which the term ascorbic acid is preceded by the name of the osone used or theoretically feasible in a synthesis by the osone hydrogen cyanide method (example *d* gluco-ascorbic acid)

**Empirical formula**

$C_6H_8O_6$

**Structural formula**




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[1] J C Drummond proposed in 1920 that the compound responsible for the prevention of scurvy be called vitamin C *Biochem J* 14 660 (1920)

[2] A Szent Györgyi and W N Haworth *Nature* 131 23 (1933)

[3] Name introduced by the Council on Pharmacy and Chemistry of the American Medical Association This organization has now changed the name to ascorbic acid

[4] A Szent-Györgyi *Biochem J* 22 1387 (1928)

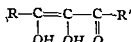
[5] R. L. Jones *Science* 68, 480 (19 8)

**Potency**

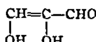
1 g = 20 000 I U

***Classification***

Ascorbic acid belongs according to its structure and properties to the class of reductones [6] originally called glucide X and later redoxin (R Wurmser 1927–1934) which are characterized by the constitution



The simplest representative of this class is the enol tartronic dialdehyde[7] [8]



All compounds of this class form reversible *oxidation reduction systems*

## 2 Chronology

Scurvy, the typical syndrome of a vitamin C deficiency, has been known for many centuries and occurred epidemically during times of war, crusades, voyages, famines, pestilences, etc (described, for example, by Euricius Cordus in 1534) Fresh vegetables have been known to provide a potent remedy for over three hundred years

1903–1913 BOLLE [9] BARTENSTEIN[10] and later HOLST and FRÖLICH[11] observed that guinea pigs could acquire scurvy just as men could and that the scurvy syndrome is a condition caused by an avitaminosis

1918–1925 ZILVA attempted the concentration of the antiscorbutic substance from lemon obtained almost pure ascorbic acid and established the important basic properties of the vitamin such as the molecular composition and size the resemblance to hexoses the instability toward oxygen especially in alkaline solution etc [12]

1920–1925 AGOPIAN[13] described methods for the isolation of practically pure ascorbic acid from cabbage

1927 On the basis of the observation that vitamin C solutions immediately after mild oxidation retained their antiscorbutic properties ZILVA[14] concluded that the

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[6] H v Euler and E Klussmann *Arkiv Kemi Mineral Geol* B11 No 12 6 pp (1933)

[7] H v Euler and C Martius *Svensk Kem Tid* 45 73 (1933) *Arkiv Kemi Mineral Geol* B11 No 14 4 pp (1933) *Ann* 505?73 (1933)

[8] T Reichstein and R Oppenauer *Helv Chim Acta* 16 988 (1933)

[9] Bolle *Z diät physik Therap* 6 354 (1903)

[10] L Bartenstein *Jahrb Kinderheilk* 61 6 (1905)

[11] A Holst *J Hyg* 7 619 (1907) A Holst and T Frölich *Ibid* 7 634 (1907) A Holst and T Frölich *Z Hyg* 72 1 (1912)

[12] A Harden and S S Zilva *Biochem J* 12 259 (1918) S S Zilva *Ibid* 17 416 (1923) 18 186 (1924) 19 589 (1925) S J B Connell and S S Zilva *Ibid* 18 641 (1924)

[13] L A Agopian F P 595 537 532 398 26 034 and 27 271

[14] S S Zilva *Biochem J* 21, 689 (1927)

antiscorbutic compound and the reducing factor were closely related although not necessarily identical

1928 SZENT GYÖRGYI[16] isolated from adrenal glands from oranges and from cabbage a strongly reducing compound the hexuronic acid

1932 The identity of vitamin C with SZENT GYÖRGYI s hexuronic acid and with ZILVA s reducing factor' was discovered by various groups of workers namely by SVIRBELY and SZENT GYORGYI[16] by WAUGH and KING[17] and by TILLMANS[18] who deduced that vitamin C can be reversibly oxidized and reduced without loss of antiscorbutic efficacy

1933 The constitution of vitamin C was established by the combined work of HAWORTH HIRST and co-workers[19] and of MICHEEL and KRAFT[20] REICHSTEIN[21] and HAWORTH[22] announced the first successful synthesis of ascorbic acid

## 3 Occurrence

Vitamin C is widely distributed over the animal and plant kingdoms It is present in all living plant cells and augmented amounts are found in all actively growing parts of higher plants Leaves and flowers for example of gladiolus nettle hip (hull and shell) and paprika, are especially rich in vitamin C Excellent sources are the citrus fruits, berries green vegetables apples, etc (see page 324)

Animal tissues contain small but definite amounts of ascorbic acid Increased amounts are found in various glands organs etc especially those of endocrine functions, for example in liver hypophysis corpus luteum, adrenal gland thymus etc Human milk contains considerable amounts of ascorbic acid blood contains small amounts (for details see pages 325 and 334)

Ascorbic acid occurs in plants predominantly as such In animal and probably also in plant tissues ascorbic acid occurs apparently in an equilibrium with its oxidized form, the dehydro ascorbic acid" The percentage present of the oxidized and the reduced forms varies considerably according to the tissue and various other physiological factors In a rabbit for example, the liver was found to contain 27% and muscles

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[16] A Szent Györgyi *Biochem J* 22 1387 (1928)

[16] J L Svirbely and A Szent Györgyi *Nature* 129 576 (193?) *Biochem J* 26 865 (1932)

[17] W A Waugh and C G King *Science* 75 357 (193 ) *J Biol Chem* 97 3?5 (1932)

[18] J Tillmans and P Hirsch *Biochem Z* 250 312 (193 )

[19] R W Herbert E G V Percival R J W Reynolds F Smith and E L Hirst *J Soc Chem Ind* 52 221 481 (1933) E G Cox and T H Goodwin *J Chem Soc* 1936 769

[20] F Micheel and K Kraft *Z physiol Chem* 222 235 (1933)

[21] T Reichstein *Nature* 132 280 (1933) T Reichstein A. Grüssner and R. Oppenauer *Helv Chim Acta* 16 561 1019 (1933)

[22] R G Ault D K Baird H C Carrington W N Haworth R W Herbert E L. Hirst, E G V Percival F Smith and M Stacey *J Chem Soc* 1933 1419 D K. Baird W N Haworth R W Herbert, E L. Hirst F Smith and M Stacey *Ibid* 1934 6?

50% of the oxidized form Fresh blood and fresh milk[23, 24, 25] contain no dehydro ascorbic acid, but only the reduced form

Besides free ascorbic acid and dehydro ascorbic acid, vitamin C occurs both in animal and in plant tissues in "combined form"[26, 27, 28] The composition of this combined form, which is called "ascorbigen," is unknown (see page 301) It has been shown[29] that, for example, livers of fresh water fish contain from 0 5–20% of the total vitamin C content in the combined form, whereas muscle tissue of the same fish contains only 0 1–0 5% Cow's milk apparently does not contain any combined ascorbic acid, but this form has been found in human milk[30] (There is considerable controversy as to whether or not the combined form actually exists,[30, 31] depending upon the technic used for the detection of the combined form)

## 4 Isolation

The isolation of vitamin C, which was achieved a number of years before the compound was recognized as a vitamin, is largely dependent upon a quick removal from its solutions Vitamin C in solution is easily oxidized This oxidation does not occur to the same extent as long as the vitamin is in its natural environment, which contains protective antioxidants (for details see page 327) All isolation procedures must, therefore, be carried out in the absence of oxygen, in the absence of copper,[32] which catalyzes the oxidation, and in the absence of light,[33] especially when riboflavin,[34] or other fluorescent compounds are present in the raw materials

Practically, the isolation consists in first preparing a water extract of the starting material (for example, from hips) or pressing the juice out of water-containing materials (for example, of citrus fruits, plant leaves,

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[23] E J Reedman *Can Pub Health J* 29 339 (1937)
[24] A Cimmino *Quaderni nutriz* 5 239 (1938)
[25] C A Knight R A Dutcher and N B Guerrant *Science* 89 183 (1939)
[26] B Ahmad *Nature* 136 797 (1935) E W McHenry and M L Graham *Ibid* 135 871 (1935) E J Reedman and E W McHenry *Biochem J* 32 85 (1938) H Scarborough and C. P Stewart *Ibid* 31 2232 (1937) *Nature* 142 40 (1938)
[27] K C Saha *J Indian Chem Soc* 16 511 (1939) B C Guha and P N Sen Gupta *Nature* 141 974 (1938) J C Pal and B C. Guha *J Indian Chem Soc* 16 481 (1939) P N Sen Gupta and B C Guha *Ibid* 16 496 (1939) B Ghosh and B C Guha *Ibid* 16 505 (1939)
[28] K Wachholder and A Okrent *Z physiol Chem* 264 254 (1940)
[29] K C Saha *J Indian Chem Soc* 16 511 (1939)
[30] K Wachholder and A Okrent *Z physiol Chem* 264 254 (1940)
[31] A Fujita and T Ebihara *Biochem Z* 301 229 (1939)
[32] A F Hess and L J Unger *Proc Soc Exptl Biol Med* 19 119 (1921)
[33] C L Arcus and S S Zilva *Biochem J* 34 61 (1940)
[34] D B Hand E S Guthrie and P F Sharp *Science* 87 439 (1938) F G Hopkins *J Soc Chem Ind* 56 934 (1937)

etc ) From these aqueous solutions impurities are precipitated with barium acetate or with neutral lead acetate and after filtration, and if necessary after the further addition of lead acetate the vitamin is pre cipitated as the lead salt by bringing the solution to a pH of about 7 6 (indicator for example bromo thymol blue) with ammonia The pre cipitate is filtered or centrifuged and decomposed in water solution with sulfuric or hydrochloric acid by bringing the solution to a pH of about 2 The sulfates or chlorides precipitated thereby are filtered off and washed and coloring matter may now be extracted, for example with *n* butyl alcohol ; The combined water solutions are concentrated under vacuum without heating or with only slight heating since temperatures above 60° C decompose the vitamin The concentrate is purified further by fractional precipitation with organic solvents such as acetone, ether, methanol, ethanol propanol, butanol, etc The pure ascorbic acid is then obtained from the filtered solution by distillation of the solvent followed by crystallization of the residue The vitamin may now be pre cipitated with acetone or with petroleum ether [35, 36] Final crystallization is carried out from methanol

A modification of this method consists in transforming the ascorbic acid into its acetone derivative (see page 295) and isolating this compound which is then decomposed by water to yield the pure vitamin C [37]

By these methods only the ascorbic acid which occurs in the free state is isolated In order to achieve a better yield the ascorbic acid present in the combined form must first be liberated This is achieved by gentle heating of a water suspension of the tissue material or of the pressed juice preferably under nitrogen followed by a reduction by means of hydrogen sulfide of the dehydro ascorbic acid originally present and formed during the operations [38]

Ascorbigen alone may be isolated from plant material by extraction of the dry material with chloroform anhydrous ether, etc , followed by water extraction From juices, for example from cabbage juice, ascor bigen is isolated by adsorption on charcoal followed by elution with a mixture of 30% chloroform and 70% absolute alcohol Further con centration may be achieved by precipitation of impurities with tungstic acid [38]

[35] W A Waugh O A Bessey and C G King *Proc Soc Exptl B ol Med* 30 1281 (1933)
[36] J L Svirbely and A Szent Györgyi *Biochem J* 27 279 (1933)
[37] E J Baumann *J B ol Chem* 89 213 (1930) E J Baumann and N Metzger *Proc Soc Exptl. Biol Med* 30 1268 (1933)
[38] B Ghosh and B C Guha *J Indian Chem Soc* 16 505 (1939)

## 5 Properties

Vitamin C crystallizes in white, odorless and colorless plates melting at 190–192° C The vitamin has a somewhat acid taste Crystallo

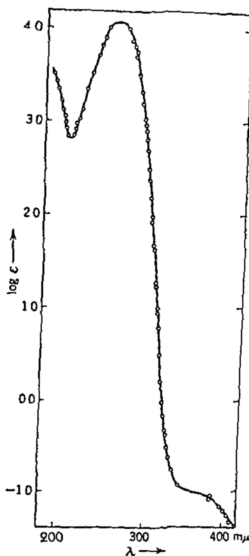



Fig 14 —Absorption spectrum of ascorbic acid in water solution (with an equimolecular amount of KCN) (H Mohler and H Lohr)

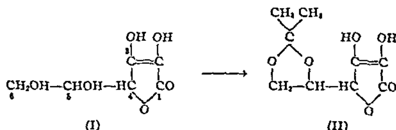
graphic and x ray measurements of the crystallized substance show that the molecule is almost completely flat [39]

[39] E G Cox *Nature* 130 205 (1932) E G Cox and T H Goodwin *J Chem Soc* 1936 769 E C Cox and E L Hirst *Nature* 131 402 (1933)

Ascorbic acid is quite soluble in water (one gram dissolves in 3 cc of water), less in alcohols (one gram in 50 cc of absolute alcohol or in about 100 cc of glycerine) and is insoluble in benzene ether chloroform fats, etc Whereas vitamin C is quite stable in crystalline form it is easily deteriorated in solution especially in the presence of air traces of metals such as copper and iron and light [40] especially in the presence of riboflavin [41] The most characteristic property of vitamin C is its strong reducing action in solution and the ease of its oxidation which is catalyzed by some metals especially by copper[42] and by silver [43] The oxidation reduction potential of ascorbic acid at pH 4 and at 35° C $E_0' = +0\ 166\ V$ [44, 45] In solution vitamin C exhibits acidic properties the dissociation constants being $pK_1 = 4\ 17$ and $pK = 11\ 57$ Vitamin C has an optical activity of $[\alpha]^{20}_D = +23°$ in water or $[\alpha]^{23}_D = +48°$ in methanol Ascorbic acid has a typical ultraviolet absorption spectrum with a maximum at 265 $m\mu$[46] ($\log \epsilon = 3\ 98$) and a small band between 350 and 400 $m\mu$[47] ($\log \epsilon = \sim 1$) (Fig 14) (see also under Determination of vitamin C Physical methods page 315) The infra red spectrum of vitamin C has also been investigated [48]

## 6 Constitution

Ascorbic acid has the empirical formula $C_6H_8O_6$ It is a monobasic acid, giving well defined salts of the type $C_6H_7O_6M$ Four of the six oxygen atoms belong to four hydroxyl groups as indicated by a determination of active hydrogen atoms Acetone condenses[49] with ascorbic acid (I) with the formation of a mono isopropylidene derivative (II)




[40] C L. Arcus and S S Zilva *Biochem J* 34 61 (1940)
[41] F G Hopkins *Compt rend t av lab Carlsberg* 22 2 6 (1938)
A F Hess and L J Unger *Proc Soc Exptl Biol Med* 19 119 (1921)
F Schlemmer B Bleyer and H Cahnmann *Biochem Z* 254 187 (1932)
[44] E G Ball *J Biol Chem* 118 219 (1937)
[45] H Borsook H W Davenport C E P Jeffreys and R C Warner *Ibid* 117 237 (1937)
[46] F P Bowden and C P Snow *Nature* 129 720 (1932)
[47] H Mohler and H Lohr *Helv Chim Acta* 21 485 (1938)
[48] E Heintz *Compt rend* 208 1813 (1939)
[49] L. v Vargha *Nature* 130 847 (1932)

hydroxyl groups is primary and that another hydroxyl group must be in $\alpha$ position to that primary hydroxyl group

Further insight into the structure of vitamin C was obtained by a number of oxidation reactions Oxidation of ascorbic acid (I) with one oxygen atom, for example, by means of air,[60] hydrogen peroxide, ferric chloride, quinones, copper acetate,[61] dichloro phenol indophenol in acid solution, iodine in acid or in neutral solution, and even by irradiation with ultra violet light[62] yields dehydro ascorbic acid (X) which can be reconverted to ascorbic acid by reducing agents such as hydrogen sulfide and hydro iodic acid The dehydro ascorbic acid, at the moment of its formation, is not an acid but a neutral compound, a lactone The lactone ring, in contrast to that of ascorbic acid, readily hydrolyzes in water solution with the formation of a free carboxylic acid group (XI) This behavior shows that ascorbic acid is not a true acid in the sense that the acidic properties are caused by a carboxylic acid group but that the acidity is caused by an enolic hydroxyl group[63] The open chain dehydro ascorbic acid cannot be reduced to ascorbic acid by means of hydrogen sulfide alone If, however, simultaneously with the $H_2S$ treatment a lactone formation is achieved, for example, by evaporation in the presence of hydriodic acid, the vitamin is formed The opening of the lactone ring is followed by complex reactions or rearrangements of the molecule, as indicated by changes of the absorption spectrum, optical rotation, etc[64] [65] Further oxidation of ascorbic acid with hydrogen peroxide yields oxalic acid[66] (XII), which suggests that a keto group is in $\alpha$ position to the carboxyl group Upon oxidation of the vitamin with sodium hypo iodite in alkaline solution, oxalic acid (XII) and *l* threonic acid (XIII) were obtained almost quantitatively[67] The latter acid was identified by methylation (XIV) followed by amidation as trimethyl *l* threonamide (XV) and by oxidation with nitric acid as *d* tartaric acid (XVI) the constitution of which was proved by the formation of dimethoxy-*d* succinamide (XVII) This sequence of oxidation reactions can only be explained when the constitution of the first oxidation product, of dehydro ascorbic acid, is that of an

[60] I Antener *Helv Chim Acta* 20 742 (1937)

[61] P Karrer H Salomon K Schöpp and R Morf *Ibid* 16 181 (1933) P Karrer H Salomon R Morf and K Schöpp *Biochem Z* 258 4 (1933) P Karrer G Schwarzenbach and K. Schöpp *Helv Chim Acta* 16 302 (1933)

[62] C L Arcus and S S Zilva *Biochem J* 34 61 (1940)

[63] E L Hirst *J Soc Chem Ind* 52 221 (1933)

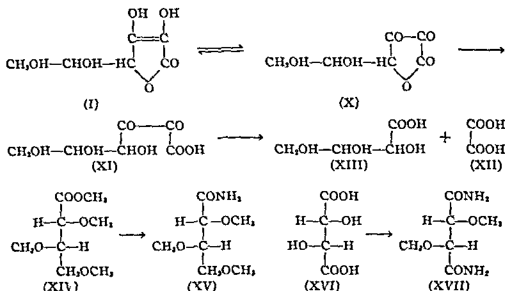
[64] E L Hirst E G V Percival and F Smith *Nature* 131 617 (1933) E L Hirst E G V Percival R W Herbert R J W Reynolds and F Smith *J Chem Soc* 1933 1270

[65] H Borsook H W Davenport C E P Jeffreys and R C Warner *J Biol Chem* 117 1 (1937)

[66] P Karrer G Schwarzenbach and K Schöpp *Helv Chim Acta* 16 302 (1933)

[67] E L Hirst *J Soc Chem Ind* 52 221 (1933)

$\alpha,\beta$ diketo compound The isolation of *l* threonic acid as a degradation product of ascorbic acid shows that the latter substance is a derivative of *l* gulose This is quite remarkable since practically all naturally occurring hexoses belong to the *d* series



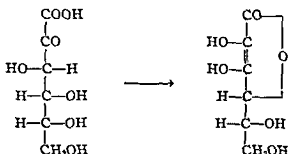

Further degradation reactions were carried out after protecting the four hydroxyl groups Thus the dimethyl ether of ascorbic acid was esterified with *p* nitro benzoyl chloride yielding a di *p* nitro benzoate which upon oxidation of the double bond gave rise to a neutral ester containing the same number of carbon atoms as the starting material[68] This proves that the molecule contains a straight chain of six carbon atoms and that the double bond is situated in a ring, or more specifically, in a lactone ring By saponification of the reaction product, oxalic acid and *l* threonic acid are obtained which shows that the position of the double bond is between carbon atoms 2 and 3 since from the six carbon atom-containing molecule a two and a four carbon atom containing substance are obtained

The nature of the ring present in the vitamin C molecule was elucidated by a degradation reaction[69] very similar to the one just described Ascorbic acid was methylated with diazomethane to the dimethyl ether (IV), which was further methylated by means of methyl iodide and silver oxide The tetramethyl ether (XVIII) obtained yielded upon ozonization a

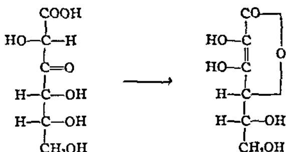
[68] F Micheel and K Kraft *Z physiol Chem* 215 215 (1933)

[69] E L Hirst E G V Percival and F Smith *Nature* 131 617 (1933) R W Herbert, E L Hirst E G V Percival R J W Reynolds and F Smith *J Chem Soc* 1933 1270

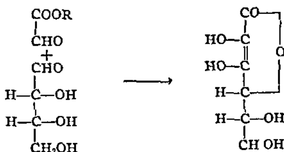
1 By Isomerization and Lactonization of 2-Keto-hexonic Acids



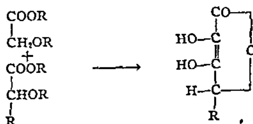

2 By Isomerization and Lactonization of 3-Keto-hexonic Acids




3 By a Benzoin Type Condensation of Two Aldehydes of Lower Molecular Weight



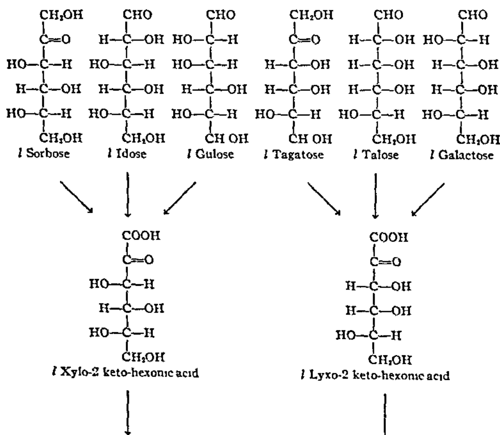

4 By Ester Condensation of $\alpha$-Oxy-acids



The various methods applicable for the synthesis of ascorbic acid according to the four outlined schemes will be shown in the following paragraphs

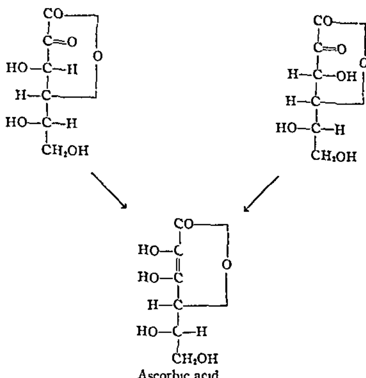
## (a) *Synthesis of Ascorbic Acid by Isomerization and Lactonization of 2 Keto hexonic Acids*

The synthesis of ascorbic acid according to this method[76] is characterized by using a 2 keto hexonic acid as intermediate which may also be called 5 keto hexuronic acid or hexusonic acid Since such an acid contains one more asymmetrical carbon atom than ascorbic acid namely, the carbon atom 3 there are two different 2 keto hexonic acids which might be converted into ascorbic acid and which have in common the stereochemical configuration at carbon atoms 4 and 5 These two compounds are called




(Formula continued on following page)

[76] T Reichstein and A Grüssner *Helv Chim Acta* 17 311 (1934)



Ascorbic acid

*l* xylo 2 keto hexonic acid and *l* lyxo 2 keto hexonic acid according to the specific configuration of the pentoses to which the hexonic acids correspond Each of these two 2 keto hexonic acids may in turn be derived from three different hexoses, namely, two aldoses and one ketose Thus, *l* idose, *l* gulose and *l* sorbose may yield *l* xylo-2 keto hexonic acid which is also called 2 keto *l* gulonic acid, and *l* talose, *l* galactose and *l* tagatose may yield *l* lyxo 2 keto hexonic acid Of these six hexoses, only *l* sorbose is readily accessible Besides this aldose also *l* gulose has been used for synthesizing vitamin C

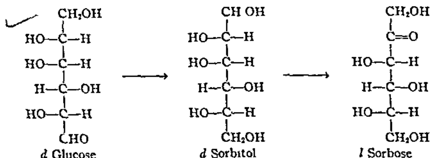
*l* Sorbose is obtained from *d* sorbitol which occurs in nature and can for example, be isolated from the fruits of mountain ash (*Sorbus aucuparia*) This hexitol is also obtained by catalytic hydrogenation of *d* glucose,[77] a process which is carried out technically on a large scale

The *d* sorbitol is converted into *l* sorbose by bacterial oxidation according to Bertrand[78] A number of bacteria are able to accomplish this reaction In practice acetic acid bacteria, are used[79]

[77] W Ipatiev *Ber* 45 3?25 (1912) W E Cake *J Am Chem Soc* 44 859 (1922) L W Floyd R Connor and H Adkins *Ibid* 54 1651 (1932) F P 694 424 I G Farbenindustrie G P 544 666

[78] G Bertrand *Bull soc chim* [3] 15 6?7 (1896) *Ann chim* [8] 3 183 227 (1904) H Schlubach and J Vorwerk *Ber* 66 1251 (1933)

[79] P A Wells L B Lockwood J J Stubbs N Porges and E A Gastrock *Ind Eng Chem* 31 1425 (1939)



*d* Glucose → *d* Sorbitol → *l* Sorbose

*l* Gulose which can also be used as starting material for the synthesis of ascorbic acid, is obtained by oxidation of starch or of *d* glucose to *d* saccharic acid, the lactone of which is reduced to *l* gulonic acid The lactone of the latter compound yields *l* gulose upon reduction [80]

The oxidation of the hexoses to the 2 keto hexonic acid namely, 2 keto-*l* gulonic acid can be carried out by a number of different methods

(1) *Via the Osone* *l* Sorbose[81] or *l* gulose[82] is converted into the phenyl hydrazone which in turn is reacted with benzaldehyde to yield the

```
   CH2OH              CHO
   |                  |
   C=O            HO—C—H
   |                  |
HO—C—H            HO—C—H
   |                  |
 H—C—OH            H—C—OH
   |                  |
HO—C—H            HO—C—H
   |                  |
   CH2OH              CH2OH
 l-Sorbose          l Gulose
```

```
   CH=N NH C6H5             CHO                  COOH
   |                        |                    |
   C=N NH C6H5              C=O                  C=O
   |                        |                    |
HO—C—H        ——→       HO—C—H      ——→      HO—C—H
   |                        |                    |
 H—C—OH                  H—C—OH               H—C—OH
   |                        |                    |
HO—C—H                  HO—C—H               HO—C—H
   |                        |                    |
   CH2OH                    CH2OH                CH2OH
  Osazone                  Osone          2 keto l gulonic acid
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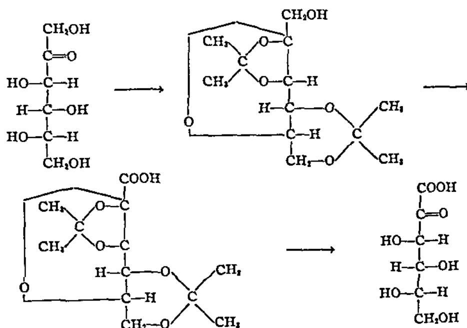
[80] P P T Sah *Ber* 69 158 (1936)

[81] P Micheel and K Kraft, *Naturwissenschaften* 22 205 (1934) F Micheel and W Lohmann *Z physiol Chem* 225 13 (1934)

[82] P P T Sah *Ber* 69 158 (1936)

corresponding osone This compound must be carefully purified in order to give finally an ascorbic acid which crystallizes well By careful oxidation with bromine[83] in aqueous solution, the osone is converted into the 2 keto *l* gulonic acid

(2) *Via the Diacetone sorbose*[84] *l* Sorbose is condensed with acetone in order to protect all groups with the exception of the primary hydroxyl group in 1 position The main reaction product is sorbose diacetone of the structure indicated below and as a by product a mono acetone sorbose is obtained The latter compound is easily separated from the diacetone derivative since only the di isopropylidene compound is soluble in typical organic solvents, such as ether The mono acetone derivative can be converted into the diacetone compound by condensation with acetone The diacetone sorbose is oxidized by means of permanganate in alkali to the 2 3,4 6 diacetone derivative of 2 keto *l* gulonic acid The free keto acid is obtained by warming the acetone compound in water This last reaction can be carried out in a yield of 82% As a by product small amounts of ascorbic acid are formed




Instead of acetone, other ketones or aldehydes, for example, methyl ethyl ketone, benzaldehyde, etc , and especially cyclic ketones, for example, cyclo hexanone may be used to protect the sorbose

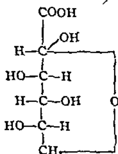
(3) *By direct Oxidation* Sorbose can be oxidized directly to 2 keto

[83] C Neuberg and T Kitasato *Biochem Z* 183 485 (1927)
[84] T Reichstein and A Grüssner *Helv Chim Acta* 17, 311 (1934)

gulonic acid since the 1 hydroxyl group is especially sensitive to oxidation This oxidation may be carried out by means of nitric acid[85] or by catalytic oxidation [86]

The 2 keto hexonic acids may also be obtained from the corresponding aldonic acids Thus *l* gulonic acid can be selectively oxidized to 2 keto *l* gulonic by means of chromic acid[87] or by chlorates in the presence of a vanadium catalyst [88]

The last step of the synthesis of vitamin C is the conversion of the 2 keto gulonic acid into ascorbic acid by lactonization This reaction does not occur voluntarily, probably because the keto acid exists mainly in the form of the stable lactol



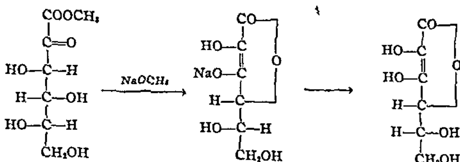

This lactonization, can, however, be accomplished by a number of different reaction conditions

1 From the free keto acid by heating in neutral acid or alkaline solution [89] The best yields are obtained in acid solution [90] The double bond produced by enolization of the keto group gives rise to a *cis* and a *trans* compound Only the *cis* form is able to undergo lactonization the *trans* form is either reconverted into the keto form or undergoes conversion into compounds different from vitamin C

2 From esters of the keto acid by the action of sodium alkoxides [91] sodium bicarbonate [92] sodium acetate [92] calcium carbonate [93] etc The reaction is said to occur also in acid solution [94] The esters of the keto acid are obtained by esterification of the free acid for example with alcohols and mineral acids or with diazo alkanes

---

[85] W N Haworth *Nature* 134 724 (1934) B P 443 901
[86] O Dalmer and K Heyns U S P 2 189 778
[87] R Pasternack and P P Regna U S P 2 153,311
[88] R Pasternack and P P Regna U S P 2 188 777
[89] Swiss P 187 933 187 934 180 810 and 188 804
[90] T Reichstein and A Grüssner *Helv Chim Acta* 17 311 (1934)
[91] Swiss P 174 208
[92] Swiss P 187 932
[93] Swiss P 180 810
[94] Swiss P 188 800 188 802 and 188 803




3 From the diacetone keto acid by heating in the presence of diluted mineral acids[95]

(b) *Synthesis of Ascorbic Acid by Isomerization and Lactonization of 3 Keto hexonic Acids Obtained by the Osone Hydrogen Cyanide Method*[96 97 98]

The synthesis of ascorbic acid according to this method involves the utilization of a 3-keto hexonic acid, which also may be called 4 keto hexuronic acid This acid can practically only be obtained by building up the molecule from a compound of five carbon atoms This is achieved by the addition of hydrogen cyanide to the osone (IV) Since specific stereochemical configurations are required for the carbon atoms 4 and 5 of ascorbic acid, there is only one pentosone (IV) with the same con figuration Theoretically, this pentosone can be obtained from three pentoses, namely, two aldoses, *l* lyxose and *l* xylose, and one ketose The latter one is still unknown

Actually, the osone (IV) has been prepared from both aldoses, *l* lyxose and *l* xylose *l* Xylose occurs in nature as part of hemicellulose and can be obtained from soft wood materials, for example, from corncobs, elder pith, beeches, sawdust, shells of coconuts, etc , by acid hydrolysis It can also be prepared from rice starch or from glucose by oxidation to *d* sac charic acid, reduction to *l* gulonic acid lactone and degradation of the latter compound to *l* xylose Perhaps the easiest method is to convert *d* sorbitol, which is technically prepared from *d* glucose, into di ethylidene sorbitol[99] by means of paraldehyde or into mono benzal sorbitol[100] by

[95] G P 641 639 recommends the use of hydrochloric acid whereas Swed P 88 094 prefers the use of sulfuric acid

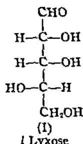
[96] T Reichstein *Nature* 132 280 (1933) T Reichstein A Grüssner and R Oppenauer *Helv Chim Acta* 16 561 1019 (1933)

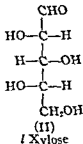
[97] T Reichstein A Grüssner and R Oppenauer *Helv Chim Acta* 17 510 (1934)

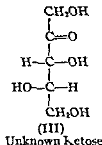
[98] R G Ault D K Baird H C Carrington W N Haworth R W Herbert E L Hirst E G V Percival F Smith and M Stacey *J Chem Soc* 1933 1419 D K Baird W N Haworth R W Herbert E L Hirst F Smith and M Stacey *Ibid* 1934 62

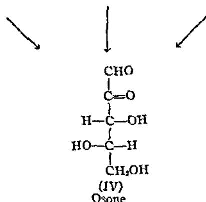
[99] Hoffman La Roche G P 627 249 H Appel *J Chem Soc* 1935 425

[100] L v Vargha *Ber* 68 18 (1935)









means of benzaldehyde followed by oxidation to di ethylidene *l* xylose or benzal *l* xylose respectively and hydrolysis to the free *l* xylose

The preparation of *l* lyxose is more complicated *d* Galactose (from pectins) is converted into the 1 2 3 4 diacetone derivative and oxidized to 1 2 3 4 diacetone *d* galacturonic acid and hydrolyzed to *d* galacturonic acid By reduction of the aldehyde group of the latter compound *l* galac tonic acid is obtained which is converted into the acid amide *l* galacton amide and finally into *l* lyxose

The oxidation of the pentoses to the osone which is called *l* xylosone is accomplished by either preparing first the osazone which is decomposed by the aid of benzaldehyde or by direct oxidation with hydrogen peroxide and ferrous sulfate as catalyst If the osazone is prepared as an inter mediate it is not necessary to start with pure crystalline pentoses

For the conversion of *l* xylosone into ascorbic acid hydrogen cyanide is added to the osone yielding the nitrile (V) which apparently enolizes and lactonizes immediately to form the cyclic imino compound (VI) since it gives none of the reactions for a nitrile group The use of potassium cyanide in the presence of calcium chloride instead of hydrogen cyanide shortens the reaction time from days to hours The cyclic imino-com pound (VI) can be isolated The isolation however, is not necessary

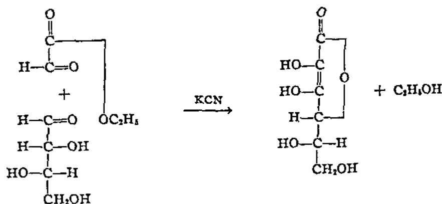
By acid hydrolysis *l* ascorbic acid is obtained, the imino group being eliminated as an ammonium salt

```
    C(=O)H                       CN                       CN
    |                            |                        |
    C=O                          CH(OK)               HO—C
    |              KCN           |                         ||
 H—C—OH          ——————→         C=O        ——————→   KO—C          ——————→
    |                            |                        |
HO—C—H                        H—C—OH                   H—C—OH
    |                            |                        |
    CH2OH                    HO—C—H                   HO—C—H
                                 |                        |
    (IV)                         CH2OH                    CH2OH
                                         (V)

          NH                                   O
          ||                                   ||
          C——                                  C——
          |   |                                |   |
     HO—C    |                            HO—C    |
          ||  O          ——————→               ||  O
     HO—C    |                            HO—C    |
          |   |                                |   |
      H—C——                                H—C——
          |                                    |
     HO—C—H                               HO—C—H
          |                                    |
          CH2OH                                CH2OH
          (VI)
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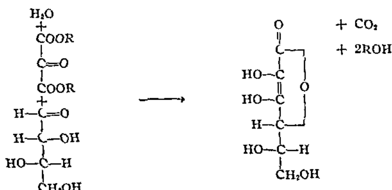
*(c) Synthesis of Ascorbic Acid by Condensation of Ethyl glyoxylate with l Threose*[101]

According to this method ascorbic acid is synthesized by a benzoin type condensation from ethyl glyoxylate and *l* threose in alkaline solution Practically, the acetylated cyano hydrine of *l* threose is used which in one operation yields ascorbic acid



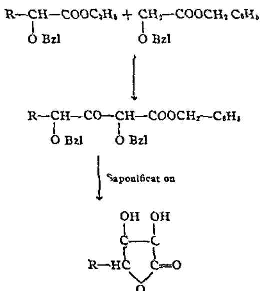

[101] B Helferich and O Peters *Ber* 70 465 (1937)

Instead of the ethyl glyoxylate ethyl mesoxylate may also be used with the same effect [102]




(*d*) *Synthesis of Ascorbic Acid by Ester Condensation of Benzoyl glycolic Acid with α Oxy acids*[103]

This method is best illustrated by the following formulas




## 8 Industrial Methods of Preparation

Vitamin C is commercially available in the crystalline form, made either by synthesis or by extraction from natural sources Since very

[102] B Helferich G P 683 954
[103] F Micheel and H Haarhoff *Ann* 545 28 (1940)

economic methods have been worked out for the synthetic route, using the 2 keto hexonic acid as intermediate, the isolation procedure from natural sources becomes less and less attractive The purity of commercial ascorbic acid is about 99%

In the canning industry special methods have been developed for essentially maintaining the natural vitamin C content of foods For example, the water used to cook vegetables is boiled before the plant material is added in order to avoid oxidation by the air dissolved in the water [104] The boiling process itself is carried out anaerobically [104] Vegetables are stored in refrigerators where the loss of vitamin C is slow, whereas at room temperature most plant materials lose their vitamin C content rapidly The freezing method for preserving food causes no essential loss of vitamin C provided the material is consumed immediately after defrosting

## 9 Biogenesis

The mechanism of the ascorbic acid formation in plants and in animals is largely unknown It seems conceivable that ascorbic acid may be produced by transformation of sugar acids of related structure such as glucuronic acid or galacturonic acid, or by total synthesis In favor of the latter hypothesis is the observation that volatile constituents of plant and animal unsaponifiable matter, lipid in nature, can serve as the precursor of ascorbic acid in the body of the rat [10] Furthermore, it has been found that the vitamin C content of livers and intestines of rats which had been subjected to extreme periods of inanition did not change significantly This suggests that the precursor of ascorbic acid was probably of endogenous origin and independent of carbohydrate intake [106]

In favor of the hypothesis that vitamin C is formed by chemical transformation of compounds of related structure is the observation that dextrose apparently increases the ascorbic acid content of slices of intestinal tissue,[107] but not of tissue slices from liver, spleen, stomach and brain, when the vitamin is determined by iodine titration Among many sugars investigated, mannose causes a greater rise of ascorbic acid than any other sugar investigated, in plant[108] and animal[109] tissue in *in vitro* and *in vivo* experiments Also *l* sorbose has been found active as precursor of vita

[104] E F Kohman W H Eddy and C Z Gurin *Ind Eng Chem* 23 1064 (1931) 25 682 (1933)
[105] R R Musulin R H Tully H E Longenecker and C G King *J Biol Chem* 129 437 (1939)
[106] C. Mentzer and G Urbain *Compt rend soc biol* 128 270 (1938)
[107] F Widenbauer and K Koschorreck *Biochem Z* 291 209 (1937)
[108] S N Ray *Biochem J* 28 996 (1934)
[109] B C. Guha and A R Ghosh *Nature* 134 739 (1934) 135 234 871 (1935) 138 844 (1936)

min C.[110] These findings, however, could not be confirmed.[111] It has recently been postulated[112] that the presence of traces of manganese is necessary for the successful synthesis of ascorbic acid from mannose and to a lesser extent from galactose and glucose in plant and in animal tissues especially in the liver. Also the inability of man and guinea pigs to produce vitamin C has been discussed in view of the low Mn concentration of their tissues.[113] It has furthermore been shown that whereas the optimum Mn concentration in *in vitro* and *in vivo* experiments with rat liver tissue is about 0.001–0.005% the concentration of manganese necessary for a successful synthesis of ascorbic acid in *in vitro* and *in vivo* experiments with guinea pig liver tissue is much higher.[113]

A correlation of available manganese with ascorbic acid production has also been found in plants. The ascorbic acid content of tomatoes from plants grown on soils low in manganese is considerably lower than from plants grown on soils with higher manganese content. In pot cultures the application of one gram of $MnSO_4 \cdot 4H_2O$ in a localized area to 15,000 g of Sassafras sandy loam soil increased the ascorbic acid content in tomato pulp from 142 to 243 mg. per liter.[114]

The various observations of increased ascorbic acid excretion following the administration of various chemicals appear to result from a slightly different chemical mechanism.[115] Particularly effective are terpene-like cyclic ketones, for example *l*- and *d*-carvone, *d,l*-piperitone, isophorone, α- and β-ionone, pulegone, thujone, camphor and neridol and somewhat less active are di-isobutyl ketone, dipropyl ketone and dimethyl acetyl carbinol. These results indicate that a stimulation of the normal ascorbic acid synthesis occurs in order to detoxify compounds which are foreign to the tissues. The increase of ascorbic acid content of rat adrenals after feeding 3-hydroxy acetonyl acetone may probably be explained similarly.[116]

For the site of ascorbic acid formation in plants and for the influence of light upon the synthesis see page 324.

## 10. Specificity

The antiscorbutic activity of vitamin C is quite specific. A number of salts are active, for example, the sodium, copper, manganese and iron

[110] G. v. Sztareczy, *Biochem. Z.* 295, 369 (1938).

[111] A. Scheunert and M. Schieblich, *Z. physiol. Chem.* 246, [illegible]72 (1937). J. R. Hawthorn and D. C. Harrison, *Biochem. J.* 31, 1061 (1937).

[112] M. N. Rudra, *Nature* 141, [illegible]03 (1938); 143, 811 (1939); *Biochem. Z.* 301, 238 (1939).

[113] M. N. Rudra, *Nature* 144, 868 (1939).

[114] J. B. Hester, *Science* 93, 401 (1941).

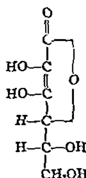
[115] H. E. Longenecker, R. R. Musulin, R. H. Tully and C. G. King, *J. Biol. Chem.* 129, 445 (1939).

[116] J. Mosonyi, *Z. physiol. Chem.* 230, 240 (1934).

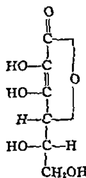
salts, and salts of organic amines, for example, of mono ethanol amine and of quinine None of the simple derivatives of ascorbic acid for example the acetone derivative,[117] the dimethyl ether, the dihydro compound or the imino ascorbic acid, are active It is *important to note* however, that the oxidized form, the dehydro ascorbic acid, in lactone form has the same activity as the non oxidized form The open chain dehydro ascorbic acid, on the other hand, is completely inactive, whereas the methyl 2 keto *l* gulonate, but not the free acid, is active,[118] presumably because the organism is able to convert this compound into ascorbic acid

Practically all the theoretically possible stereoisomers and simple homologs of ascorbic acid have been prepared and tested for antiscorbutic activity From the results of these experiments it is concluded that for antiscorbutic activity a *d* configuration is necessary at carbon atom 4, that a side chain must be attached to this carbon atom, and that in the side chain a hydroxyl group is necessary in 5 position All hydroxyl groups must be free

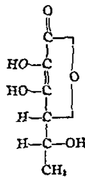
The number of active compounds is therefore, quite limited They are *d arabo ascorbic acid*[119, 120, 121, 122] (*iso ascorbic acid*) (about 1/20 of the activity of ascorbic acid), 6 desoxy *l* ascorbic acid[123] (about 1/3 of the activity of ascorbic acid), *l* rhamno ascorbic acid[124] (about 1/5), *l* gluco ascorbic acid[125] (about 1/40), *l* fuco ascorbic acid[125] (about 1/50) and *d* gluco



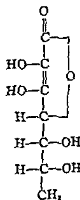
*l* Ascorbic acid



*d* Arabo-ascorbic acid



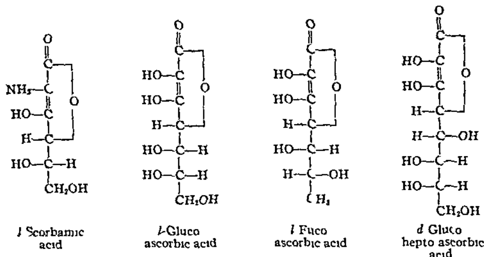
6 Desoxy *l* ascorbic acid



*l* Rhamno-ascorbic acid

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117 F Micheel and T Moll *Z physiol Chem* 219 253 (1933)
118 T Reichstein and V Demole *Festschrift für F C Barell* Basel 1936
119 T Reichstein A Grüssner and R Oppenauer *Helv Chim Acta* 17 510 (1934)
120 H Ohle H Erbach and H Carls *Ber* 67 324 (1934)
121 K Maurer and B Schiedt *Ibid* 66 1054 (1933) 67 1239 (1934)
122 O Dalmer and T Moll *Z physiol Chem* 222 116 (1933)
123 H Müller and T Reichstein *Helv Chim Acta* 21 273 (1938)
124 T Reichstein L Schwarz and A Grüssner *Ibid* 18 353 (1935)
125 T Reichstein and V Demole *Festschrift für E C Barell* Basel 1936



*l* Scorbamic acid — *l*-Gluco ascorbic acid — *l* Fuco ascorbic acid — *d* Gluco hepto ascorbic acid

hepto ascorbic acid[125] (about $^1/_{100}$) *l* Scorbamic acid has also been reported[126] to show antiscorbutic activity Of all the compounds *l* ascorbic is the most potent

Vitamin C is the only naturally occurring antiscorbutic compound However, another substance has been isolated from adrenal cortex[127] of beef, which is said to show faint antiscorbutic activity and which possesses the reducing and general chemical properties of ascorbic acid The composition was found to be C 25 86%, H 5 97% N 5 97% P 0 55% The chemical and physical data of this compound do not permit any conclusion to be drawn as to the purity or the constitution of the compound It might well be that a form of ascorbigen, a combined ascorbic acid, was isolated Another possibility would be that ascorbic acid can be chemically bound in a way similar to the vitamins of the B group to complex molecules containing phosphoric acid thus forming part of enzyme systems Any definite opinion about the existence of a second naturally occurring antiscorbutic substance must be withheld until further experimental data become available

## 11 Determination

### (a) *Physical Methods*

**Spectroscopical Method** The determination of the intensity of the characteristic absorption spectrum in water solution at 265 mμ, for ex

[126] F Micheel and R Mittag *Naturwissenschaften* 25 158 (1937) *Z physiol Chem* 247 34 (1937)
[127] E Ott K Krämer and W Faust *Z physiol Chem* 243 199 (1936)

ample, before and after destruction of the vitamin, has been advocated [128] Since vitamin C in solution is quickly destroyed, the determination must be carried out rapidly An addition of reducing agents has been recommended The position of the absorption band is somewhat different in various solvents *In alcohol for example, the maximum is at 245 mμ* The position of the band is, furthermore, a function of the pH With decreasing pH the maximum shifts toward shorter wave lengths [129] The best method of determining ascorbic acid is to use a water *solution of the* vitamin to which an equimolecular amount of potassium cyanide as stabilizer has been added (Fig 14 on page 294) [130, 131]

**Polarographic Method** Determination of the oxidation potential of ascorbic acid in acid solution, for example, in extracts *from fruits or* vegetables, has given quite satisfactory results [132]

### (*b*) *Chemical Methods*

Chemical methods of determining vitamin C are generally used today replacing almost entirely the biological methods of earlier days The chemical methods are mostly based on the great reducing ability of ascorbic acid Since besides ascorbic acid the naturally occurring dehydro ascorbic acid also exhibits vitamin C activity, care must be taken to include the non reducing dehydro ascorbic acid in determinations of vitamin C in natural products It should, furthermore, be observed that apparently varying amounts of vitamin C are chemically bound to protein materials, in which combination the ascorbic acid shows no reducing action

**Titration with Iodine** The vitamin C content of *pure solutions can* be determined by titration with 0 01 *N* iodine solution This method proved to be inadequate for the determination of ascorbic acid in natural products, since they contain other reducing substances besides vitamin C and since the color of such products interferes with the determination of the end point of the iodine titration

**Titration with 2,6-Dichloro-phenol-indophenol** The determination of vitamin C by titration with 2,6 dichloro phenol indophenol (see formula p 317) originally suggested by Tillmans [133] is the most widely used method today A great number of different modifications have been

[128] E B Robertson *J Soc Chem Ind* 53 277 (1934) A Chevallier and Y Choron *Compt rend soc biol* 124 453 (1937)

[129] B Starzynski *Bull Acad Pol Sci Letter* A 1937 460

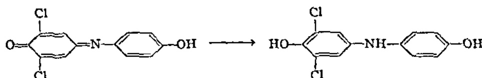
[130] R A Morton *The Application of Absorption Spectra to the Study of Vitamins and Hormones* London 1935

[131] H Mohler and H Lohr *Helv Chim Acta* 21 485 (1938)

[132] K Schwartz *Z anal Chem* 115 161 (1939) T Osternd *Tek Ukeblad* 86 216 (1939)

[133] J Tillmans *Z Untersuch Lebensm* 54 33 (1927)

proposed to overcome the various insufficiencies resulting from the determination of vitamin C in the presence of other plant or animal materials The indophenol dye oxidizes besides ascorbic acid for example sulfhydryl compounds[134] thiosulfate,[135] pyridinium compounds[136] (medication) the reduced forms of nicotinic acid derivatives and of riboflavin[137] etc In organic and organic ferrous and ferric compounds also interfere with the determination[138] In beer yeast malt mold etc organic reducing substances are found which react exactly like ascorbic acid toward the indophenol indicator These compounds are sugar derivatives formed by alkali Known examples of these are reductic acid[139] and reductone[140]




2 6 Dichloro phenol indophenol

A certain amount of vitamin C in solution is often present in the dehydro form which is not determined by the indophenol titration In order to include the dehydro form it is necessary to convert this form first into ascorbic acid This is carried out by hydrogen sulfide at pH 4–7 followed by an elimination of the excess hydrogen sulfide by blowing nitrogen through the solution The results obtained by this method, however, are not always trustworthy The following method has therefore been suggested (1) to convert the total vitamin C content into the dehydro form for example by passing through Norite or by ascorbic acid oxidase (2) to determine the amount of reducing substances left (3) to reduce the dehydro compound with hydrogen sulfide and (4) to titrate the vitamin C with indophenol[141] The difference between the value from the second titration and the value of the first titration gives fairly reliable results of the total amount of ascorbic acid present unless, as in urine, substances are present which are capable of slow reduction with hydrogen sulfide to indophenol reducing substances[142] This difficulty can how

134 A Emmerie *Biochem J* 28 ?68 (1934)

135 M van Eekelen *Acta Brev a Neerland Physiol Pharmacol Microbiol* 4 137 (1934) M Heinemann *Ibid* 6 67 (1936)

136 C F Cannon and T McGovern *Proc Soc Exptl Biol Med* 38 267 (1938)

137 W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)

138 K. P Basu and M C Nath *J Indian Chem Soc* 15 133 (1938)

139 T Reichstein and R Oppenauer *Helv Chim Acta* 17 390 (1934)

140 B K Nelson and C A Browne *J Am Chem Soc* 51 830 (1929) H v Euler and C. Martius *Ann* 505 73 (1933)

141 A Emmerie and M van Eekelen *Biochem J* 28 1151 (1934) 30 ?5 (1936)

142 H Scarborough and C. P Stewart *Ibid* 31 2–32 (1937)

ever, be overcome by appropriate use of a photoelectric colorimeter, by following the progress of the titration as a function of time and by extrapolation of the results obtained [143, 144, 145] The determination of vitamin C in fruits and vegetables may be carried out by any of these methods with a fair degree of accuracy Since the amount of the dehydro form in the living plant is generally insignificant (it is significant, however, in stored materials), approximate values can be obtained by direct titration if the fresh material is extracted sufficiently rapidly with a strong acid, for example, acetic acid,[146] trichloro acetic acid,[147] metaphosphoric acid,[148] oxalic acid,[149] etc , to diminish the oxidation of vitamin C by the oxidase present in the plant cells and to exclude the titration of glutathione present, and if the titration is performed immediately thereafter

Some other modifications have also been proposed Thus, it has been suggested[150] to extract the tissues in the cold with sulfuric acid or with phosphoric acid, to reduce the extract, for example, with hydrogen sulfide, cadmium, zinc, aluminum, palladium, chromium or titanium at a pH of about 4 5, and to titrate with indophenol before and after an addition of copper sulfate Since the copper ions oxidize preferentially the ascorbic acid, the difference of the two titrations corresponds to the ascorbic acid content

The accuracy of the determination of vitamin C in extracts of animal origin is somewhat limited Liver, for example, may give values up to 20% too high Metaphosphoric acid is particularly useful for the extraction of animal tissues since it also deproteinizes the extracts [151] Foreign reducing materials may be removed with lead acetate[152] or with mercuric acetate [153] The latter, however, also oxidizes ascorbic acid to a considerable extent to dehydro ascorbic acid

Special methods have been worked out to determine the ascorbic acid concentration in blood plasma[154] and in milk,[155] which involve the use of oxalic acid either alone or in mixture with metaphosphoric acid Dehydro ascorbic acid is determined by reduction with hydrogen sulfide

[143] R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
[144] O A Bessey *Ibid* 126 771 (1938)
[145] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[146] R Strohecker and R Vaubel *Angew Chem* 49 666 (1936)
[147] T W Birch L J Harris and S Ray *Biochem J* 27 590 (1933)
[148] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[149] B Willberg *Z Untersuch Lebensm* 76 128 (1938)
[150] M Ott *Angew Chem* 54 170 (1941)
[151] A Fujita and T Ebihara *Biochem Z* 290 172 (1936)
[152] V A Deviatnin and V M Doroshenko *Ibid* 280 118 (1935)
[153] A Emmerie and M Eekelen *Biochem J* 28 1151 (1934) 30 25 (1936)
[154] R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
[155] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)

The sensitivity of the determination of ascorbic acid with indophenol is quite satisfactory and in questionable cases for example, in cases of prevalent turbidity, the results obtained are checked by adding a known amount of ascorbic acid to the solution

The end point of the titration with the indophenol dye may be determined by visual observation or by the aid of a colorimeter[156] or a photoelectrometer[157, 158, 159] The latter method is especially accurate since a decrease in concentration of the dye, produced by the addition of a vitamin C containing extract of insufficient concentration to cause complete reduction of the indicator,[160] can be measured The titration of very dark solutions may be carried out by extracting the excess dye after the complete oxidation of the vitamin[161]

All these methods as described include only the determination of ascorbic acid and of dehydro ascorbic acid but not the determination of the combined form ascorbigen although experimental evidence has been presented that the ascorbic acid is liberated from ascorbigen by extraction with metaphosphoric or sulfo salicylic acid[162, 163] In order to determine the total true ascorbic acid content of plant or animal material the vitamin must be liberated from its carrier An aqueous suspension of the material is heated while passing hydrogen sulfide through the solution, and after removal of the excess hydrogen sulfide by carbon dioxide or nitrogen the solution is titrated, for example before and after a treatment with ascorbic acid oxidase The transformation into the dehydro compound by means of the specific enzyme or by some other means such as copper ions appears to be necessary, since during the heating process in the presence of hydrogen sulfide an appreciable amount of reducing compounds other than vitamin C is formed which are capable of being oxidized by the indophenol dye[164]

**Titration with Methylene-blue** Ascorbic acid reduces methylene blue in the presence of light to the leuco compound This reaction has repeatedly been used[165, 166] and advocated for the quantitative deter

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[156] M Ott *Angew Chem* 51 537 (1938)
[157] K A Evelyn *J Biol Chem* 115 163 (1936) K A Evelyn H T Malloy and C Rosen *Ibid* 126 645 (1938)
[158] T Gutbe and K K Nygaard *J Soc Chem Ind* 57 1195 (1938)
[159] R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
[160] O A Bessey *Ibid* 126 771 (1938)
[161] F Siebert *Dissertation* Frankfurt a Main 1931
[162] K Wachholder and A Okrent *Z physiol Chem* 264 254 (1940)
[163] A Fujita and T Ebihara *Biochem Z* 301 229 (1939)
[164] P N Sen Gupta and B C Guha *J Indian Chem Soc* 16 549 (1939)
[165] H Lund and H Lieck *Nature* 137 784 (1936)
[166] I Gál, *Ibid* 138 799 (1936)

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Some other modifications have also been proposed Thus, it has been suggested[150] to extract the tissues in the cold with sulfuric acid or with phosphoric acid to reduce the extract, for example with hydrogen sulfide cadmium, zinc aluminum, palladium, chromium or titanium at a pH of about 4 5, and to titrate with indophenol before and after an addition of copper sulfate Since the copper ions oxidize preferentially the ascorbic acid, the difference of the two titrations corresponds to the ascorbic acid content

The accuracy of the determination of vitamin C in extracts of animal origin is somewhat limited Liver, for example, may give values up to 20% too high Metaphosphoric acid is particularly useful for the extraction of animal tissues since it also deproteinizes the extracts [151] Foreign reducing materials may be removed with lead acetate[152] or with mercuric acetate [153] The latter, however, also oxidizes ascorbic acid to a considerable extent to dehydro ascorbic acid

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[143] R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
[144] O A Bessey *Ibid* 126 771 (1938)
[145] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[146] R Strohecker and R Vaubel *Angew Chem* 49 666 (1936)
[147] T W Birch L J Harris and S Ray *Biochem J* 27 590 (1933)
[148] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[149] B Willberg *Z Untersuch Lebensm* 76 128 (1938)
[150] M Ott *Angew Chem* 54 170 (1941)
[151] A Fujita and T Ebihara *Biochem Z* 290 172 (1936)
[152] V A Deviatnin and V M Doroshenko *Ibid* 280 118 (1935)
[153] A Emmerie and M Eekelen *Biochem J* 28 1151 (1934) 30 25 (1936)
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ıI Ott *Angew Chem* 51 537 (1938)

. A Evelyn *J B ol Chem* 115 163 (1936) K A Evelyn H T Malloy and C Rosen *Ibid* 45 (1938)

` Guthe and K K Nygaard *J Soc Chem Ind* 57 1195 (1938)

ꝛ L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)

) A B ss y *Ibid* 126 771 (1938)

ꝛ S ebert *Disse tation* Frankfurt a Main 1931

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143 R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
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145 W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
146 R Strohecker and R Vaubel *Angew Chem* 49 666 (1936)
147 T W Birch L J Harris and S Ray *Biochem J* 27 590 (1933)
148 W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
149 B Willberg *Z Untersuch Lebensm* 76 128 (1938)
150 M Ott *Angew Chem* 54 170 (1941)
151 A Fujita and T Ebihara *Biochem Z* 290 172 (1936)
152 V A Deviatnin and V M Doroshenko *Ibid* 280 118 (1935)
153 A Emmerie and M Eekelen *Biochem J* 28 1151 (1934) 30 25 (1936)
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I Ott *Angew Chem* 51 537 (1938)
A Evelyn *J B ol Chem* 115 163 (19‿6) K A Evelyn H T Malloy and C Rosen *Ibid* 45 (1938)
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Fuj ta and T Ebihara *Biochem Z* 301 ??9 (1939)
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[144] O A Bessey *Ibid* 126 771 (1938)
[145] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[146] R Strohecker and R Vaubel *Angew Chem* 49 666 (1936)
[147] T W Birch L J Harris and S Ray *Biochem J* 27 590 (1933)
[148] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)
[149] B Willberg *Z Untersuch Lebensm* 76 128 (1938)
[150] M Ott *Angew Chem* 54 170 (1941)
[151] A Fujita and T Ebihara *Biochem Z* 290 172 (1936)
[152] V A Devjatnin and V M Doroshenko *Ibid* 280 118 (1935)
[153] A Emmerie and M Eekelen *Biochem J* 28 1151 (1934) 30 25 (1936)
[154] R L Mindlin and A M Butler *J Biol Chem* 122 673 (1938)
[155] W W Woessner C A Elvehjem and H A Schuette *J Nutrition* 18 619 (1939)

**Vanadium Test**[178] Vitamin C yields a blue color, which later changes to green with a reagent prepared from vanadium pentoxide and sulfuric acid

**Barac's Azo Test**[179] Diazotized sulfanilic acid is reduced by the action of ascorbic acid and forms the orange compound



$$HO_3S—C_6H_4N{=}NC_6H_4—SO_3H$$

**Sulfanilamide Test**[180] A solution of sulfanilamide sodium nitrate, sulfo salicylic acid and urea is mixed with a solution of ascorbic acid, and $\alpha$ dimethyl naphthyl amine is added The color developed is compared with the color of the same mixture without the vitamin

**Selenous Acid Test**[181] Selenous acid solutions produce an orange red color with ascorbic acid in solution

**Gold-trichloride test**[181] The ability of ascorbic acid to reduce gold trichloride has been used for the determination of vitamin C

**Mercuric Chloride Test**[182] Mercuric chloride is precipitated from a solution of mercurous chloride by the addition of ascorbic acid

**Pitarelli's Test with Cupric Sulfate and Ammonium Thiocyanate**[182] A white precipitation occurs upon the addition of cupric sulfate and ammonium thiocyanate and a green color develops upon the addition of further amounts of ammonium thiocyanate to ascorbic acid solutions

**Szent-Gyorgyi's Reaction with Ferrous Sulfate** A dark violet color develops upon addition of a ferrous sulfate solution to an alkali solution of vitamin C The color is bleached upon reduction with hyposulfite and can be restored by air oxidation

**Test According to Tauber**[183] An acetic acid solution of ascorbic acid yields a blue color when first a solution of ferric cyanide and then a solution of ferric sulfate and phosphoric acid is added

**Furfural Test**[184] Vitamin C upon boiling with hydrochloric acid forms furfural which is determined with the known aniline phloroglucinol or resorcinol test Pentoses are included in the values obtained, whereas hexoses and glucuronic acid have a comparatively small furfural producing capacity[185]

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[178] A T Freses *Bol soc quim Peru* 4 22 (1938)
[179] G Barac *Compt rend soc biol* 126 61 (1937)
[180] J V Scud and H D Ratish *Ind Eng Chem Anal Ed* 10 4 0 (1938)
[181] A Emmerie *Acta Brevia Neerland Physiol Pharmocol Microbiol* 4 141 (1934)
[182] E Pittarelli *Biochim terap sper* 22 100 (1936)
[183] H Tauber *Mikrochemie* 17 111 (1935)
[184] J H Roe *Science* 80 561 (1934)
[185] G E Youngburg *J Biol Chem* 73 599 (1927)

mination of vitamin C The vitamin is extracted from plant or animal material by means of trichloro acetic acid[167] or sulfo salicylic acid [168]

**Quantitative Determination by Means of Folin's Reagent** [169] Vitamin C, extracted from animal or plant material by metaphosphoric acid, has been claimed to be oxidized specifically by mono iodo acetic acid and Folin s re agent,[170] which consists of a solution of molybdenum free sodium tungstate in dilute orthophosphoric acid to which a small amount of bromine water is added A blue color is produced which is measured in a color imeter This reaction is not very specific and is given, for example, by other dihydroxy compounds and by phenols

A similar color is obtained by the addition of sodium tungstate in sulfuric acid to a solution of the vitamin in metaphosphoric acid followed by the addition of alkali [171]

**Determination by Means of Molybdenum-phosphotungstic acid** [172] This reagent produces with vitamin C a violet color which is measured colorimetrically

**Test According to Giri Using Ferricyanide and Ammonium Molybdate** [173] Ascorbic acid in trichloro acetic acid solution reduces potassium ferricyanide which upon further addition of ammonium molybdate yields a red brown precipitate Vitamin C solutions from natural sources are purified by the addition of mercuric acetate which precipitates pigments tannins, glutathione cysteine etc

**Phosphomolybdic Acid Test** [174, 175] Phosphomolybdic acid produces a blue color in acid solution with ascorbic acid

**Bachstez-Cavallini Reaction with Uranyl-acetate** [176] [177] Uranyl ace tate in slightly alkaline solution produces with ascorbic acid a brown color discharged by the alkali followed by precipitation of sodium uranate This reaction serves as a test for differentiating vitamin C from isoascorbic acid, since with the latter compound only a brownish color develops but no precipitation occurs

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[167] E Martini and A Bonsignore *Biochem Z* 273 1,0 (1934) *Boll soc ital biol sper* 9 388 (1934)

[168] W Quensel and K Wachholder *Z physiol Chem* 231 65 (1935) K Wachholder and H H Podesta *Ibid* 239 149 (1936)

[169] A Fujita and T Eb hara *Biochem Z* 290 182 (1936)

[170] O Folin *J Biol Chem* 106 311 (1934)

[171] A Fujita D Iwatake and T Mijata *Biochem Z* 277 ⁹06 (1935)

[172] N Bezssonoff *Z Vitaminforsch* 5 193 (1936)

[173] K V Giri *Microchemie* 23 ⁹83 (1938)

[174] H Tillmans and P Hirsch *Z Untersuch Lebensm* 63 2 13 (19 2)

[175] N Bezssonoff *B ll soc chim biol* 16 1107 1133 1160 (1934) H v Euler and D Burström *Biochem Z* 283 153 (1936)

[176] M Bachstez and G Cavallini *Z physiol Chem* 228 2ɔ (1934)

[177] A Emmerie *Acta Brevia Neerland Physiol Pharmocol Microbiol* 4 141 (1934)

### (d) *Biological Methods*

Although chemical and to a small extent also physical methods are replacing more and more the biological determinations of vitamin C the biological tests maintain their place as the ultimate and most correct method of determining vitamin C

Guinea pigs are the only animals which can be used When they are kept on a vitamin C free diet, they develop scurvy within two to three weeks Vitamin C determination tests are carried out using either the principle of the preventive method that is determining the smallest amount which prevents macroscopic signs of scurvy for example decrease in weight, or the principle of the curative method [193] that is determining the smallest amount which will cure guinea pigs showing definite signs of scurvy Another method is the histological examination of the teeth of guinea pigs [194] In a scorbutic tooth the odontoblast layer is disorganized, the predentine is calcified and an irregular layer of bone is formed between the odontoblasts and the predentine

## 12 Standards

By definition

1 I U (International Unit) vitamin C = 0 05 mg of pure ascorbic acid[195]
= 1 U S Pharmacopoeia Unit
1 Sherman Unit = 0 5–0 6 mg vitamin C
= 1 minimum protective dose (guinea pig)

The minimum protective unit for guinea pigs has been defined as the smallest amount of ascorbic acid which when ingested daily prevents in guinea pigs of about 200 g weight at least for 60 days the occurrence of the syndromes of a vitamin C deficiency

The Standard Reference as recommended by the Permanent Commission on Biological Standardization of the League of Nations in 1931 was 0 1 ml of freshly squeezed lemon juice which was defined as 1 International Unit This standard was changed in 1934 following the availability of the pure material One Unit of the new standard corresponds in activity approximately to one Unit of the older Standard

## 13 Physiology of Plants and Microorganisms

Vitamin C is a necessary growth factor for plants and microorganisms This has been demonstrated[196] on pea embryos which have been isolated

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[193] L J Harris J I Mills and J R M Innes *Lancet* 2 235 (1935) L J Harris and S N Ray *Biochem J* 27 2006 (1933)

[194] J A Höjer *Brit J Exptl Path* 7 356 (1926) K. M Key and G K Elphick *Biochem J* 25 888 (1931)

[195] *Quart Bull Health Organization League Nations* 3 428 (1934)

[196] J Bonner and D Bonner *Proc Natl Acad Sci U S* 24 70 (1938) D Glick *Z physiol Chem* 245 211 (1937) S von Hausen *Biochem Z* 288, 378 (1936)

A modification of the furfural test is the *osazone furfural method* This has especially been adapted for the quantitative determination of vitamin C in urine The vitamin is oxidized to dehydro ascorbic acid by passing through Norite, and separated as a dinitro phenyl osazone The nitro groups are reduced with stannous chloride followed by hydrolysis of the osazone and the dehydro ascorbic acid obtained is determined by conversion into furfural, etc Another possibility is to titrate the dinitro phenyl osazone with titanium chloride in acid solution [186]

***Cacotheline Test*** [187] Cacotheline (nitro brucin) produces in hydrochloric acid solution a lilac color with vitamin C and other reducing agents

**Determination of Vitamin C by Its Oxidation to Oxalic Acid** Vitamin C is oxidized in acid solution with permanganate After destroying the excess oxidizing agent with hydrogen peroxide, the oxalic acid produced is determined [188]

**Prussian Blue Method** An acid ferricyanide solution is easily reduced by vitamin C and converted into Prussian blue by known methods The amount of blue is measured with a colorimeter

The *determination of dehydro ascorbic acid in the presence of ascorbic acid can be accomplished* by reaction with 2,4 dinitro phenyl hydrazine The osazone of the dehydro ascorbic acid precipitates and can be determined gravimetrically or by titration with titanium chloride

The rough *determination of vitamin C* in cells is carried out by the silver nitrate staining technic of Bourne, Giroud, Leblond and associates

## (c) *Biochemical Methods*

**Ascorbic Acid Oxidase Method** [189] This method is based on the ability of ascorbic acid oxidase to oxidize, preferentially ascorbic acid This oxidase however, is not specific enough to give trustworthy results in the presence of other natural products [190] especially in the presence of extracts from animal tissues which contain, besides ascorbic acid, other compounds capable of reducing methylene blue [191] It has, furthermore, been observed that a special preparation of oxidase from pumpkin did not react with vitamin C in human urine, spinal fluid and cow s milk [19]

[186] L Espil and L Genevois *Bull soc chim* 5 1532 (1938)
[187] L Rosenthaler *Z Vitaminforsch* 7 126 (1938)
[188] M Paget and R Berger *Compt rend soc biol* 129 960 (1938)
[189] H Tauber and I S Kleiner *J B ol Chem* 110 559 (1935)
[190] G A Snow and S S Zilva *Biochem J* 32 1926 (1938)
[191] K Wachholder and A Okrent *Z physiol Chem* 264 254 (1940)
[192] N Bezssonoff and H Vertruyen *Compt rend soc biol* 128 407 (1938)

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from their seeds and thereby from their natural nutritional resources Dry seeds do not contain any demonstrable amounts of this vitamin during their inactive rest period, but contain some unknown precursor which is converted into ascorbic acid immediately upon the beginning of germination Tubers, such as the potato tuber, on the other hand, contain appreciable amounts of vitamin C

The beneficial effect of ascorbic acid on higher plants can also be demonstrated in other cases There is a considerable species difference, for example, eggplant shows no response, tobacco plants, however, gain considerably in growth upon administration of ascorbic acid solutions [197]

It is not known where vitamin C is formed in higher plants but it is found regularly in high amounts in all growing parts Adult parts contain some vitamin C but parts which have turned into wood do not contain any ascorbic acid at all Haw contains the vitamin in the hull and shell, but not in the seeds [198] The highest amount is found usually in flowers and in leaves Petals contain more ascorbic acid than pistils, stamens and calyces [199]

Light seems to have a beneficial effect upon the production of ascorbic acid in plants [200] This finding might probably be linked with the observation that red and violet flowers generally contain more active material than white and yellow flowers [201] The concentration of ascorbic acid in plant leaves is also a function of the light received and fluctuates considerably during the day, the maximum being reached, for example in potato leaves in the forenoon [202]

The mechanism of the ascorbic acid action in plants is largely unknown The conception of a participation in the oxidation reduction systems of the living plant will be discussed with the corresponding action in animal tissues (See page 326 )

The physiology of vitamin C in microorganisms differs considerably with the species Thus some bacteria need vitamin C and are apparently able to synthesize it [203] This question has especially been studied on lactic acid bacteria and it has been found that some strains show a definite growth response to vitamin C added to the culture medium,[204] while others are re

[197] R Dennison *Science* 92 17 (1940)
[198] H Winckelmann *Hippokrates* 9 714 (1938)
[199] H Mituda *J Agar Chem Soc Japan* 14 1228 (1938)
[200] M E Reid *Am J Botany* 25 702 (1938)
[201] H Mituda *J Agr Chem Soc Japan* 14 1228 (1938)
[202] A M Smith and J Gillies *Biochem J* 34 1312 (1940)
[203] G Bourne and R Allen *Australian J Exptl Biol Med Sci* 13 165 (1935)
[204] O Rahn and C P Hegarty *Proc Soc Exptl Biol Med* 38 218 (1938) O Rahn C P Hegarty and R E Deuel *J Bact* 35 547 (1938) A Sartory R Sartory and J Meyer *Compt re d* 206 1414 (1938) J G Davis and J McClemont *J Dairy Research* 10 94 (1939)

tarded and some apparently do not respond either way Vitamin C is synthesized for example, by *Bacillus prodigiosus*[205] as proved by chemical and biological determination It has also been demonstrated that some protozoa need an external supply of ascorbic acid for optimal growth [206]

## 14 Animal Physiology

Of all the vitamins vitamin C has received the widest attention in a large number of various investigations relating to different phases of physiological interest Nevertheless the knowledge about the general physiology, the metabolism and the mechanism of the vitamin C action is still fragmentary and the fundamentals of these subjects are still unknown

### (a) *Metabolism*

Ascorbic acid is absorbed by the tissues of the intestinal tract [207] principally of the small intestine After oral ingestion of vitamin C the vitamin level in blood plasma rises to a maximum within about 1 5 hours but occasionally, for example after an intake of strawberries or cauliflower a considerably longer period of time elapses before the vitamin level in the blood is increased [208] In any event the increase is only temporary The vitamin is transported with the blood throughout the entire organism and excess amounts are secreted in the urine[209] where it appears mostly in the free form, but to a small extent also in a combined form [210] Ascorbic acid given intravenously or subcutaneously raises temporarily the vitamin C content of the blood, but is excreted within one to three hours [211 212] More prolonged effects are obtained if the vitamin is injected intramuscularly This, however, causes sloughing due to the acidity of the vitamin The preferred method in cases of necessary parenteral administration is to inject salts for example, sodium salt or salts of organic amines for example of mono ethanol amine [213]

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[205] K H Busing and F Peters *Biochem Z* 304 134 (1940)

[206] M Lwoff *Compt rend* 206 540 (1938) *Compt rend soc biol* 130 406 (1939) R Cailleau *Compt rend soc biol* 127 861 (1938) 131 964 (1939) 138 319 (1939)

[207] S S Zilva *Biochem J* 29 100 (1935)

[208] E N Todhunter *Am Inst Nutrit on Abstracts J Nutritio* 21 12 (1941)

[209] L J Harris and S N Ray *Lancet* 1 71 (1935) H v Euler and M Malmberg *Biochem Z* 279 338 (1935)

[210] B C Guha and P N Sen Gupta *Nat e* 141 874 (1938) H Scarborough and C P Stewart *Biochem J* 31 2 32 (1937) *Nature* 142 40 (1938)

[211] F E Hawley and D J Stephens *Proc Soc Exptl Biol Med* 34 8 4 (1937) M van Eekelen and M Heinemann *J Clin Invest gat on* 17 293 (1938)

[212] E P Ralli G J Friedman and M Kaslow *Proc Soc Exptl Biol Med* 36 52 (1937) I S Wright A Lilienfeld and E MacLenathen *Arch Inte nal Med* 60 264 (1937) J M Faulkner and F H L Taylor *J Clin Investigat on* 17 69 (1938)

[213] E L Lozner F J Pohle and F H L Taylor *New England J Med* 220 987 (1939)

Tissues and body fluids contain various amounts of this vitamin Normal human blood plasma contains about 1 2 mg ascorbic acid in 100 cc [214] There are no real storage organs for this vitamin, although some organs contain increased amounts Among these the adrenal gland contains the most It has been shown, however, that, for example in guinea pigs the amount of ascorbic acid in this gland cannot be increased, even by feeding excess amounts of vitamin C [215] Generally, tissues of high metabolic activity have the highest vitamin C content Thus, young tissues contain more vitamin C than older tissues, as has been shown on the thymus[216] and the corpus luteum [217] Elderly people generally have less vitamin C reserves than young people Vitamin C is secreted in the milk Cow s milk contains on the average about 22 mg /l Human milk contains several times more vitamin C than cow s milk (about 75 mg /l ), since babies, but not calves, need an external supply of this vitamin Colostrum contains somewhat more vitamin C [218] The ascorbic acid secretion in cow's milk varies somewhat with the season The highest amount has been found in the late summer or early fall [219] Ascorbic acid is also at times excreted in the sweat [220] The main excretion is through the urine, as stated before The excretion occurs within four to six hours following ingestion [221] Some ascorbic acid is also excreted in the feces [22]

## (b) *Physiological Action*

The most obvious property of ascorbic acid is the reversible oxidation and reduction capacity, and much speculation and experimental work arose from the idea of correlating this behavior with the mechanism of the vitamin action It seems established that under physiological conditions the reversibility of the reducing capacity of ascorbic acid exists, although this reversibility is only partial in *in vitro* experiments The instability of the oxidized form dehydro ascorbic acid, even in the intact cell, is probably the reason for the fact that the organism needs a relatively greater amount of this vitamin than of the other vitamins

[214] A F Abt C J Farmer and I M Epstein *J Pediatrics* 8 1 (1936) D J Stephens and E E Hawley *J Biol Chem* 115 653 (1936)

[215] G Mouriquand M Dauvergne and V Edel *Compt rend* 209 1023 (1939)

[216] D Glick and G R Biskind *J Biol Chem* 113 27 (1936) 114 1 (1936)

[217] B C Guha and P N Sen Gupta *Nature* 141 974 (1938)

[218] F Schlemmer B Bleyer and H Cahnmann *Biochem Z* 254 187 (1932)

[219] A D Holmes F Tripp E A Woelffer and G H Satterfield *J Nutrition* 17 187 (1937)

[220] A Lilienfeld I S Wright and E MacLenathen *Proc Soc Exptl Biol Med* 35 184 (1936) R E Bernstein *Nature* 140 684 (1937)

[221] E E Hawley and D J Stephens *Proc Soc Exptl Biol Med* 34 854 (1936) M van Eekelen and M Heinemann *J Clin Investigation* 17, 293 (1938)

[222] H Chinn and C. J Farmer *Proc Soc Exptl Biol Med* 41 561 (1939)

It has been postulated that there exist special enzyme systems which take care of both the oxidation and the reduction of ascorbic acid Thus an enzyme has been found in blood[223] and in plant juices[224] which reduces dehydro ascorbic acid' On the other hand glutathione has been shown to be the most effective reductant and protective agent for vitamin C in the living animal and in some plant cells [225, 226] Besides glutathione other compounds with fixed sulfhydryl groups exert reducing capacities upon the oxidized form of vitamin C Also certain purines such as xanthine, uric acid and theophylline but not caffeine and theobromine, and creatinine but not creatine have been shown experimentally to protect the vitamin against oxidation [227] The opposite reaction, the oxidation of ascorbic acid is much easier to demonstrate and is carried out by a special enzyme ascorbic acid oxidase The existence of this enzyme in plants is established,[228, 229, 230] but its occurrence in animal tissues is questionable Ascorbic acid oxidase has the constitution of a copper protein[230] and is specific for the stereochemical configuration of *l* ascorbic acid [231] Also, various polyphenylases are able to oxidize ascorbic acid in plants [232] The search for the nature of the ascorbic acid oxidizing enzyme in animal tissues has brought forward many experimental evidences which partly support and partly contradict the conception of the constitution of the enzyme as a copper protein It seems plausible to assume that a number of different oxidation reduction systems act on ascorbic acid in the living organism Thus conclusive evidence has been presented that ascorbic acid is rapidly oxidized by cytochrome oxidase plus cytochrome *c* [233] The question of the nature of the mechanism of the ascorbic acid action should therefore be separated from any discussion of the reversible oxidation reduction action of this vitamin until specific reactions of physiological importance in various organs cells, body fluids etc , have been established

A number of observations along these lines have been made but it is impossible at the moment to correlate these findings and to decide which ones

[223] J H Roe and G L Barnum *J Nutrition* 11 359 (1936)

[224] E M Crook and F G Hopkins *Biochem J* 32 1356 (1938)

[225] H Borsook H W Davenport C E P Jeffreys and R C Warner *J Biol Chem* 117 237 (1937)

[226] F G Hopkins and E J Morgan *Biochem J* 30 1446 (1936)

[227] K. V Giri and P V Krishnamurthy *Nature* 147 59 (1941)

[228] A Szent Györgyi *J Biol Chem* 90 385 (1931)

[229] Z I Kertesz R B Dearborn and G L Mack *Ibid* 116 717 (1936)

[230] P L Lovett Janison and J M Nelson *J Am Chem Soc* 62 1409 (1940)

[231] H Rosenberg *Skand Arch Physiol* 76 119 (1937)

[232] D Keilin and T Mann *Proc Roy Soc (London)* B125 187 (1938) F Kubowitz *Biochem Z* 299 3 (1938)

[233] D Keilin and E F Hartree *Proc Roy Soc (London)* B125 171 (1938) E Stotz C J Harrer M O Schultze and C. G King *J Biol Chem* 122 407 (1938)

of all fibrous tissues and non epithelial cement substances [254-255] During avitaminosis, these fibrils or collagen are not formed These phenomena are in close relationship to the disturbance of the calcium metabolism during times of vitamin C depletion, which affects growth and maintenance of bones and teeth and which sometimes appears similar to that observed during vitamin D deficiency Also the phenomena of hemorrhagic lesions associated with vitamin C depletion appear to be connected with this basic function of the ascorbic acid Experimental studies have also shown the particular presence of vitamin C in Golgi cells in the cerebral cortex and the cord, the function of which cells is to bring neighboring cells into relation to each other, and in the mitochondria, which form an essential part of the cytoplasm [256]

Of particular interest is the reaction of the human organism toward ascorbic acid administration in cases of various poisonings It has already been pointed out in the section on the Biogenesis of vitamin C (page 312) that the organism of ascorbic acid producing animals reacts to the adminis tration of chemicals, especially of ketones, by increasing the ascorbic acid excretion In human beings beneficial effects of large doses of vitamin C have been recorded in cases in which the vitamin may act as a detoxicant Cases have become known in which toxic doses of drugs, for example, *l* tyrosine,[257] chemicals [258] for example lead[259] and arsenic[260] compounds, benzene, [261] etc , toxins [262] virus and substances producing anaphylaxis,[263] etc , have had no toxic effect when administered simultaneously with vitamin C It seems that ascorbic acid combines with some of these substances and is excreted in such combination Similar detoxification effects are observed toward diphtheria toxins [264] [265] tuberculosis,[266] and

[254] S B Wohlbach and P R Howe *Arch Path* 1 1 (1926) S B Wohlbach *Am J Path* 9 689 (1933) *J Am Med Assoc* 108 7 (1937)

[255] A v Jeney and E Torö *Arch path Anat (Virchow's)* 298 87 (1936)

[256] A Giroud and C P Leblond *Anat Record* 68 113 (1937) G Bourne and R. Allen *Australian J Exptl Biol Med Sci* 13 165 (1935)

[257] R R Sealock and H F Silberstein *Science* 90 517 (1939)

[258] M Vauthey *Ann maladies vénériennes* 32 98 (1937) *Ann dermatol syphilol* 8 568 (1937)

[259] H N Holmes E J Amberg and K Campbell *Science* 89 3?2 (1939) *J Lab Clin Med* 24 1119 (1939)

[260] M B Sulzberger and B L Oser *Proc Soc Exptl Biol Med* 32 716 (1935) F E Cormia *Can Med Assoc J* 36 392 (1937) S Landfisch *Polska gas lek* 16 575 (1937) I Dainow *Presse méd* 45 1670 (1937)

[261] A Meyer *Z Vitaminforsch* 6 83 (1937)

[262] C W Jungeblut *J Immunol* 33 203 (1937)

[263] F Diehl *Klin Wochschr* 18 956 (1939)

[264] A Sigal and C G King *J Pharmacol Exptl Therapy* 59 468 (1937) 61 1 (1937)

[265] C G King and M L Menten *J Nutrition* 10 129 141 (1935) C K Greenwald and E Harde *Proc Soc Exptl Biol Med* 32 1157 (1935)

[266] W W Jetter and T S Bumbalo *Am J Med Sci* 195 362 (1938) M N Rudra *Current Sci* 8 210 (1939)

many other infectious diseases[267] During such times the amount of ascorbigen excreted in the urine is increased[268] Guinea pigs which died from diphtheria intoxications showed reduced vitamin C content of the suprarenals[269] whereas no significant differences were found between the vitamin C content of the suprarenals of animals injected with a sublethal dose of diphtheria toxins and those of normal animals[270] Bacterial toxins can cause a decrease of as much as 50 to 85% of the normal vitamin C content of the adrenals[271]

In this connection the correlation of vitamin C to the complement is of importance The complement is a thermolabile protein substance in the blood serum which destroys bacteria and other cells The complement exhibits a reversible oxidation reduction potential the maintenance of which is to a great extent a function of the ascorbic acid content of the blood serum[272]

Vitamin C has also been linked with the amino acid metabolism It has, for example been shown *in vitro* that dehydro ascorbic acid dehydrates amino acids for example, leucine, with the formation of ammonia and strongly reducing acidic compounds, probably keto acids[273]

A close relationship of vitamin C to the carbohydrate metabolism has for example, been demonstrated in the case of guinea pigs in which the capacity for metabolizing glucose[274] or dextrose[275] is moderately lowered in the prescorbutic and scorbutic stage of vitamin C deficiency Vitamin C increases blood sugar in cases of hypoglycemia and in prolonged usage tends to prevent hypoglycemia In schizophrenics given insulin shock treatment, the vitamin raises the blood sugar and enables the patient to be revived more quickly than by sugar administration Vitamin C is thus a factor in carbohydrate metabolism This has also been shown in a disease in which there is a disturbance of muscle glycogen metabolism[276]

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267 D Perla and J Marmorston *Arch Path* 23 683 (1937)

268 B Ghosh *J Ind an Chem Soc* 16 241 (1939)

269 E Harde *Compt rend* 199 618 (1934) C W Jungeblut and R. L Zwemer *Proc Soc Exptl. Biol Med* 32 1229 (1934–35)

270 C C Torrance *J Biol Chem* 132 575 (1940)

271 C M Lyman and C G King *J Pha macol Exptl Therapy* 56 209 (1936) L J Harris R Passmore and W Pagel *Lancet* 2 183 (1937) C C Torrance *J Bact* 33 645 (1937)

272 E E Ecker L Pillemer D Wertheimer and H Gradis *J Immunol* 34 19 (1938) E E Ecker L Pillemer J J Griffiths and W P Schwartz *J Am Med Assoc* 112 1449 (1939)

273 H v Euler P Karrer and F Zehnder *Helv Ch m Acta* 17 157 (1934)

274 A S gal nd C. G King *J Biol Chem* 116 489 (1936)

275 A Sigal and C. G King *J Pharmacol Exptl Therapy* 59 468 (1937) 61 1 (1937)

276 E Wille *Deut med Wochschr* 65 1117 (1937)

It is also suggested[277] that vitamin C, copper and protective substances, like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases

### (c) *Relation of Vitamin C to Other Vitamins, Hormones, Etc*

As stated previously in the general chapter on vitamins, there is no synergistic or antagonistic action of one vitamin to another [278] Vitamin C has the special faculty of detoxifying a large variety of different compounds including toxic doses of other vitamins In animals which have the power of synthesizing their own supply of ascorbic acid the observation has generally been made that the amount produced is reduced at times of low vitality Therefore in rats fed a vitamin A free diet the ascorbic acid content of the heart and probably also of the kidney and the thymus is significantly reduced [279] Similar reductions in the ascorbic acid concentration in various tissues and endocrines have been observed in vitamin $B_1$ and riboflavin deficiencies, but no noteworthy changes occurred as a result of $B_6$ avitaminosis [279]

A close relationship of vitamin C to various hormones is noted since during avitaminosis a decreased hormone secretion is observed from those glands which normally contain high concentrations of vitamin C, for example, pituitary, pancreas, adrenal, thyroid, liver, intestinal wall [280] In particular, it has been observed that vitamin C is necessary for the utilization of the adrenal cortex hormones, especially for the salt metabolism controlled by these hormones [281] There seems to exist an antagonism between vitamin C and thyroxine Administration of this hormone reduces the vitamin C content of liver, adrenals, etc This effect can probably be linked with the property of ascorbic acid of detoxifying harmful compounds

The relationships of vitamin C to various enzymes and to traces of metals, especially to manganese and copper, have already been discussed

## 15 Avitaminosis and Hypovitaminosis

The state of hypovitaminosis which is quite common among human beings is characterized by impairment of physiological functions Guinea

[277] K V Giri *Biochem J* 33 309 (1939)

[278] P E Simola *Acta Soc Med Fenn Duodecim* 16 No 3 1 (1933) *Ber ges Physiol exptl Pharmakol* 83 312 A Scheunert *Naturwissenschaften* 28 297 (1940)

[279] B Sure R M Theis and R T Harrelson *J Biol Chem* 129 245 (1939)

[280] A Sigal and C G King *Ibid* 116 489 (1936)

[281] J L Svirbely *Ibid* 116 543 (1936)

pigs during hypovitaminosis are very sensitive to injury from diphtheria and other toxins[282, 283] and toward infectious diseases[284] In humans, a depletion of the vitamin C reserves occurs during many diseases, for example during fever,[285] tuberculosis[286] etc During avitaminosis the vitamin C level in blood serum and in the urine is lowered and the normal metabolic activity is decreased More severe deficiency causes sore and swollen joints edema, shortness of breath and a decline in weight The clinical symptoms of vitamin C avitaminosis are summarized under the term scurvy and are mainly characterized by hemorrhagic conditions The actual place of these is largely influenced by growth and stress[287] Thus in the growing human hematomas, steoporosis bone pains etc, occur The site of hemorrhages in adults is determined mainly by physical stress During avitaminosis bones cease to grow and the normal junctions are replaced by connective tissue which contains calcified cartilages, but is devoid of osteoid tissue This phenomenon can be seen in roentgenograms The enamel cementum and most predominantly, the dentine change in structure, become resorbed and porotic and the newly formed material is of inferior strength (osteodentine) The gingiva the gum of the jaws and surrounding the teeth swells up becomes spongy and bleeds easily In severe cases hemorrhagic lesions are also observed in muscles eyes (cataract[288]) and skin (lesions of the acne type[289]) Furthermore, a typical anemia of scurvy develops Concomitant signs of a vitamin C depletion are atrophy of the glands of internal secretion and of the lymphatic tissues The clinical symptoms of vitamin C deficiency in infants are known under the name Moller Barlow s disease

Vitamin C shortage in the organism is most dangerous in cases of bone fractures[290] and wound healing[291] which are not cured rapidly and properly unless enough vitamin C is available Cases have also been reported[292] showing that subnormal dark adaptation of the eye may be caused by vita

[282] A Sigal and C G King *J Pharmacol Exptl Therapy* 59 468 (1937) 61 1 (1937)

[283] C G King and M L Menten *J Nutrition* 10 129 141 (1935) C K Greenwald and E Harde *Proc Soc Exptl Biol Med* 32 1157 (1935)

[284] D Perla and J Marmorston *Arch Path* 23 683 (1937)

[285] K. Daum K Boyd and W D Paul *Proc Soc Exptl Biol Med* 40 1?9 (1939) Falke *Klin Wochsch* 18 818 (1939)

[286] W W Jetter and T S Bumbalo *Am J Med Sci* 195 36? (1938) M N Rudra *Current Sci* 8 210 (1939)

[287] G Dalldorf *J Exptl Med* 50 293 (19 9)

[288] H Scarborough and C P Stewart *Biochem J* 31 2232 (1937)

[289] M Lo za *Minerva Medica* 30 ?35 (1939)

[290] H Henke *Deut Z Chirurgie* 245 530 (1935)

[291] W Aron *Die Nährschäden des Kindes* Berlin 1928 T H Lanman and T H Ingalls *Ann Surg* 105 616 (1937) M Taffel and S C Harvey *Proc Soc Exptl Biol Med* 38 418 (1938) A W Allen *Intern Abstract Surg* 69 111 (1939)

[292] M S Kimble and E S Gordon *J Biol Chem* 128 LII (1939)

min C depletion at times when enough vitamin A and riboflavin were ingested However a possible relationship of vitamin C to certain forms of cataract has been denied [293]

Administration of vitamin C in human therapy is also indicated in cases of excessive bleedings (with the exception of hemophilia),[294] rheumatic fever[295] and arthritis, anaphylaxis, drug hypersensitivity, certain forms of allergy, lead and arsenic poisoning,[296] and Addison's disease in order to decrease the degree of pigmentation of the skin [297] In cases of disturbances of the gastrointestinal tract and especially in gastric and duodenal ulcers,[298] vitamin C deficiencies occur quite often due to intertestinal destruction of the vitamin or to poor absorption Thus chronic scurvy is often diagnosed as pyorrhea [299] In such cases the vitamin is administered parenterally, preferably by intramuscular injection [300] Since vitamin C is also a factor in carbohydrate metabolism, an administration of this vitamin has been suggested in those illnesses in which there is a disturbance of the glycogen metabolism, in hypoglycemia, etc [301]

In guinea pigs, typical changes of the female sex organs occur The development of the follicles becomes greatly retarded and no corpora lutea develop at all If the state of avitaminosis endures over a prolonged length of time, these changes cannot be repaired by the administration of vitamin C

## (a) *Clinical Test Methods*

Clinically the detection of a vitamin C deficiency is of importance especially when a state of hypovitaminosis is suspected The following methods are now generally used

**Blood Test** The ascorbic acid content of normal human blood plasma is about 1 2 mg per 100 cc, but this value decreases considerably upon a

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[293] J Urbanek *Klin Monatsbl f Augenh* 101 671 (1938)

[294] E Vogt *Münch med Wochschr* 82 263 (1935) H O Neumann *Klin Wochschr* 15 368 (1936) D K Miller and C P Rhoades *J Clin Investigation* 15 462 (1936)

[295] J F Rinehart *J Lab Clin Med* 21 597 (1936) J F Rinehart L D Greenberg M B Olney and F Choy *Arch Internal Med* 61 552 (1938) M A Abbasy N G Hill and L J Harris *Lancet* 2 1413 (1936) M A Abbasy L J Harris and P Ellman *Ibid* 2 181 (1937)

[296] H N Holmes E J Amberg and K. Campbell *Science* 89 322 (1939) *J Lab Clin Med* 24 1119 (1939)

[297] T Cornbleet *Arch Dermatol Syphilol* 35 471 (1937) J F Wilkinson and C A Ashford *Lancet* 2 967 (1936) A F Abt and C J Farmer *J Am Med Assoc* 111 1555 (1938)

[298] A B Rivers and L A Carlson *Proc Staff Meetings Mayo Clinic* 12 383 (1937) G Bourne *Brit Med J* 1 560 (1938) B Portnoy and J F Wilkinson *Ibid* 1 554 (1938)

[299] D Weisberger *J Conn State Med Soc* 1 492 (1937)

[300] M Vauthey *Rev Gastroenterol* 6 337 (1939)

[301] E Wille *Deut med Wochschr* 65 1117 (1937)

itamin C deficient diet In the prescorbutic state the content is about 8 mg and by the time clinical symptoms of scurvy become evident the itamin C content of blood plasma is around 0 5 mg [302, 303] When vitamin ; is administered in excess amounts the blood level may approach a value f 2 00 mg per 100 cc

The actual determination of vitamin C in blood is carried out, for example, by titration with the indophenol indicator [304] It is necessary to deproteinize the blood by means of a strong acid, for example trichloro acetic cid, tungstic acid[305] or metaphosphoric acid,[306] and thereafter to separate he clear blood plasma This method has also been adapted for microork[307] The Prussian blue method [308] the methylene blue[309] and some of he other methods described under chemical methods of determining vitamin C" (page 316) have occasionally been used Since the precipitation of rotein material sometimes carries ascorbic acid along, it has also been uggested to titrate blood serum directly with indophenol in the presence of ydrochloric acid [310] It is necessary to determine the vitamin C content of blood serum immediately after the separation of the red blood cells since h vitamin C content decreases rapidly [311] On the other hand, these determinations of vitamin C in the blood plasma are of fair accuracy if arried out properly, since no dehydro ascorbic acid is present in fresh lood, but is found only as an artefact

Urine Test The daily urinary output of vitamin C in avitaminotic umans is greatly diminished in comparison to the normal output Clinical eterminations are made on the urine of patients before and after an oral or referably after an intramuscular administration of moderate doses of itamin C Since a normal human being needs about 25–50 mg of ascorbic cid daily, the response of the urinary excretion to the administration of his amount is determined [312, 313] The urinary output of ascorbic acid of

[302] J M Faulkner and F H L Taylor *J Clin Investigation* 17 69 (1938) I S Wright *Ann Internal Med* 12 516 (1938) H Lund *Klin Wochschr* 16 1085 (1937) G A Goldsmith and G F llinger *Arch Internal Med* 63 531 (1939) F N Todhunter and R C Robbins *J Nutrition* 19 33 (1940)

[303] C J Farmer and I M Epstein *J Pediatrics* 8 1 (1936)

[304] R L Mindlin and A. M Butler *J Biol Chem* 122 673 (1938)

[305] F H L Taylor D Chase and J M Faulkner *Biochem J* 30 1119 (1936)

[306] C J Farmer and A F Abt *J Pediatrics* 8 1 (1936) *Proc Soc Exptl Biol Med* 38 399 (1938)

[307] C J Farmer and A F Abt *Proc Soc Exptl Biol Med* 34 146 (1936)

[308] H Ta ber and I S Kleiner *J Biol Chem* 110 559 (1935)

[309] H Lund and H Lieck *Klin Wochschr* 16 555 (1937) H Wahren *Ibid* 16 1496 (1937) A lmby and T Wirth *Ibid* 16 748 (1937)

[310] N Berend and M Fischer *Biochem Z* 291 2?1 (1937)

[311] R J Kassan and J H Roe *J Biol Chem* 133 579 (1940)

[312] W B Belser H M Hauck and C A Storvick *J Nutrition* 17 513 (1939)

[313] L. J Harris and S N Ray *Lancet* 1 71 (1935) H E Archer and G Graham *Ibid* 1 710 (1936) — Sloan *J Lab Clin Med* 23 1015 (1938)

healthy people varies considerably and can be influenced by a change in the acid base balance of the food consumed [314]

The exact actual determination of the total urinary excretion of vitamin C, comprising ascorbic acid, dehydro ascorbic acid and ascorbic acid bound to protein material, is rather difficult It has, for example, been suggested to use the titration with indophenol in various modifications[315] or with methylene blue,[316] the colorimetric determination with molybdo phosphotungstic acid[317] or to use the osazone furfural method [318, 319]

A certain small amount of ascorbic acid is said to be excreted in combined form [320] This amount is not included in all the results of ascorbi acid determination, unless this part of the total ascorbic acid content is liberated from the combined form During many diseases, particularly during times of fever, diphtheria, etc , the amount of the combined form is considerably increased [321]

**Skin Capillary Fragility Test** One of the first signs of a state of ub clinical scurvy (and of a vitamin P deficiency) is the considerably lowered capillary resistance [3, 9, 323] A relative quantitative picture of the vitamin C depletion of a patient can be obtained by measuring the fragility This is done for example, in the compression test, by pinching the skin with the finger for one minute and investigating the number and severity of the petechiae (hemorrhagic spots) produced A more exact effect is obtained by applying pressure with a sphygmomanometer[324] which is inflated to a pressure below the diastolic pressure of the pulse A suction method can also be used, applying negative pressure The average resistance of human skin is about 30 cm Hg but varies considerably with different parts of the skin

**Roentgenographic Examination of Bones** [325] Since a slight hypovitaminosis causes no changes in the bone structure only more severe cases can be detected by roentgenographic studies On the other hand, scurvy may be manifest in the skeleton without other clinical symptoms The patho

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[314] E E Hawley J P Frazer L L Button and D L Stevens *J Nutrition* 12 215 (1936)

[315] A Jezler and W Niederberger *Klin Wochschr* 15 710 (1936) J Gander and W Niederberger *Münch Med Wochschr* 83 1386 2074 (1936) H Kaiser *Süddeut Apoth Ztg* 1936 No 85

[316] H Lund *Klin Wochschr* 16 1085 (1937)

[317] N Bezssonoff and E Stoerr *Z Vitaminforsch* 5 193 (1936)

[318] J H Roe and J M Hall *J Biol Chem* 128 329 (1939)

[319] L Espil and L Genevois *Bull soc chim* 5 1532 (1938)

[320] S Banerjee *J Indian Chem Soc* 17 463 (1940)

[321] B Ghosh *Ibid* 16 241 (1939)

[322] G Dalldorf *J Exptl Med* 58 289 (1931) *Am J Diseases Children* 46 794 (1933)

[323] G F Göthlin *Skand Arch Physiol* 61 2?5 (1931)

[324] I S Wright and A Lilienfeld *Arch Internal Med* 57 241 (1936)

[325] E A Park H G Guild D Jackson and M Bord *Arch Disease Childhood* 10 265 (1935)

ogical symptoms are encountered in the peripheral region and the end of he shaft of the long bones

**Adrenal Cortex Examination** For autopsy purposes a rough deter- nination of the vitamin C content is made by soaking the opened gland in a ilver nitrate solution In cases of deaths caused by vitamin C depletion ery little silver precipitation occurs

**The Intradermal Test** This method is based on the observation that a solution of dichlor phenol indophenol injected under the epithelium de colorizes[326] It has been estimated that a discoloration time of a given amount of the dye of five minutes indicates saturation with vitamin C and ten minutes or more indicates hypovitaminosis[327] This test however, proved to lack sufficient specificity for clinical work[328]

## 16 Hypervitaminosis

A state of vitamin C hypervitaminosis is unknown It has been impossi ble to produce any toxic symptoms with guinea pigs by feeding excess amounts of this vitamin and no increase in the vitamin C content of the organs over their normal levels could be detected[329] No toxic signs were observed in human beings who were given doses of from 1 to 6 g orally or intravenously[330] Occasional vagotonic symptoms are attributed to idio syncrasy or drug sensitivity[331] Ingestion of ascorbic acid has a slight diuretic effect,[332] less than that caused by theobromine, but greater than the diuresis induced by digitalis[333] In animals the blood pressure rises somewhat upon injections of ascorbic acid[334]

## 17 Requirements[335]

Of the entire living world only man the other primates the guinea pig and a few microorganisms (see under Physiology, page 324) are known to require an external supply of vitamin C All other animals and plants also need vitamin C but are able to synthesize it that is ascorbic acid is a hormone for all these organisms Guinea pigs need from 1 to 2 mg of

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[326] H Rotter Nat r 139 717 (1937)
[327] B Portnoy and J F Wilkinson *Lancet* 1 3 8 (1938)
[328] H G Poucher and C H Stubenrauch *J Am Med Assoc* 111 302 (1938)
[329] G Mouriquand M D uvergne and V Edel *Compt rend* 209 1093 (1939)
[330] A F Abt and C J Farmer *J Am Med Assoc* 111 1555 (1938)
[331] F Widenbauer *Klin Wochsch* 15 1158 (1936)
[332] M A Abbasy *Biochem J* 31 339 (1937)
[333] W Evans *Lancet* 1 308 (1938)
[334] M Kasahara and R Kawamura *Klin Wochschr* 16 1543 (1937)
[335] S L Smith *J Am Med Assoc* 111 1753 (1938)

ascorbic acid daily The average optimal intake for human beings is about 50–100 mg The fact that infants require vitamin C should be especially emphasized, since cow's milk does not contain sufficient amounts and human milk is given usually only over a relatively short period of time and its vitamin C content decreases after a few weeks Infants need 3 to 8 mg per kilogram of body weight per day children about 5 to 7 5 mg, adults 0 7 to 1 6 mg, and aged people need about 3 to 5 mg of ascorbic acid per kilogram of body weight per day Pregnant and nursing women need 5 to 10 mg per kilogram of body weight per day The recommended daily allowances for ascorbic acid as established by the Food and Nutrition Board of the National Research Council will be found on page 613

It is interesting to note the relatively high requirements of this vitamin on the weight basis compared to the daily needs of man and animals for the other vitamins The order of magnitude is about 1000 times the weight of some of the other vitamins required (Compare however, the high requirements of choline) A normal diet contains, however, adequate amounts of vitamin C

# THE GROUP OF
# VITAMINS D

# THE GROUP OF VITAMINS D[1]

## 1 Nomenclature and Survey

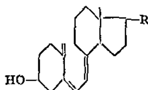
Vitamins D are usually called simply "vitamin D". The individual members of this group have not been properly named as yet. Provisionally they are called vitamin $D_2$, $D_3$, $D_4$, etc. Vitamin $D_2$ is called "calciferol" in England and has been given the name "viosterol" by the Council on Pharmacy and Chemistry (United States).

**Historical Names,** *now abandoned*

Rachitamin
Rachitasterol[2]
Antirachitic vitamin

**Chemical formulas**

General formula for vitamins D *




Vitamin $D_2$ (activated ergosterol, calciferol, viosterol) R =



Vitamin $D_3$ (activated 7-dehydrocholesterol) R =



Vitamin $D_4$ (activated 22-dihydroergosterol) R =



Vitamin $D_5$ (activated 7-dehydrositosterol) R =



(* *See page 342 for footnote*)

[1] See also C. I. Read, H. C. Struck and I. E. Steck, *Vitamin D*, Chicago, 1939.
[2] R. L. Jones, *Science* 68, 480 (1928).

## 2 Chronology

1822 Trousseau in his book on *Clinical Medicine* recommended cod liver oil as a remedy for the cure of rickets [3] and J Sniadecki inferred in his book *On the Physical Education of Children* the curative effect of sunlight [4] but their view points did not become widely known

1890 Palm[5] associated through geographical studies the incidence of rickets with deficiency of sunlight

1905 Buchholz[6] apparently cured cases of human rickets with artificial light

1906 Hopkins[7] suggested that rickets is caused by the absence of an accessory food stuff" Funk[8] (1914) corroborated this concept of the origin of rickets

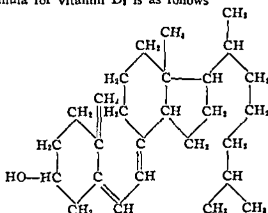
1913 Raczynski[9] established the beneficial influence of sunlight on the calcium assimilation of puppies

1919 Mellanby[10] discovered the nutritional importance of animal fats for the normal calcification of bones by raising dogs affected with rickets through special diet and by curing the animals with animal fats He concluded that the antirachitic factor' is either identical with fat soluble A (vitamin A) or has a natural distribution somewhat similar to fat soluble A

Huldschinsky[11] proved on the basis of x ray studies that severe rickets in children can be cured by the light of a mercury vapor quartz lamp Not until this report was published was the importance of sunlight appreciated

1920–1921 Sherman and Pappenheimer[12] and McCollum and Simmonds[13] succeeded in inducing rickets in rats by special diet

* The formulas of the vitamins D as indicated on p 341, are abbreviated The complete chemical formula for vitamin $D_2$ is as follows




[3] A Trousseau *Clinical Medicine* Philadelphia 1882
[4] W Mozolowski *Nature* 143 121 (1939)
[5] T A Palm *Practitioner* 45 271 321 (1890)
[6] E A Park *Physiol Rev* 3 106 (1923)
[7] F G Hopkins *Analyst* 31 385 (1906) *J Physiol* 44 425 (1912)
[8] C Funk *Die Vitamine* Wiesbaden 1914
[9] J Raczynski *Compt rend assoc intern pediat* 1913 308
[10] E Mellanby *J Physiol* 52 LIII (1919) *Lancet* I 407 (1919)
[11] K Huldschinsky *Deut med Wochschr* 45 712 (1919) *Z orthop Chir* 39 426 (1919–20)
[12] H C Sherman and A M Pappenheimer *Proc Soc Exptl Biol Med* 18 193 (1920–21) *J Exptl Med* 34 189 (1921)
[13] E V McCollum and N Simmonds *J Biol Chem* 47 111 139 175 207 235 507 (1921) See also V Korenchevsky *Brit Med J* 547 (1921) *Special Rept Sci Med Research Council* No 71 (1922)

1921 HESS and UNGER[14] proved that the earlier observations concerning the curative effect of sunlight on patients with rickets were correct They also reported the rickets preventive effect of sunlight for rats on a rickets producing diet

1922 ZUCKER PAPPENHEIMER and BARNETT recognized that the unsaponifiable fraction of fish liver oils contained the antirachitic factor [15]

1922 McCOLLUM and his co-workers[16] established experimentally the distinctive properties of vitamin A and the antirachitic factor At the same time HUME[17] and independently SHEETS and FUNK[18] found that light would not cure the effects brought about by an insufficiency of vitamin A
HESS and GUTMAN[19] expressed the opinion that the curative effect brought about on patients with rickets by either cod liver oil or light was fundamentally the same

1924 STEENBOCK and associates[20] and independently HESS[21] found that antirachitic potency could be induced in foods by ultraviolet irradiation

1925 The work of HESS WEINSTOCK and HELMAN [22] of STEENBOCK and BLACK [23] and of ROSENHEIM and WEBSTER[24] indicated that the sterol fraction of foodstuffs could be made antirachitically active by irradiation although it was not active in itself
McCOLLUM named the antirachitic material vitamin D

1925–1926 SCHLUTZ and MORSE[25] postulated the possibility and ROSENHEIM and WEBSTER [26] HEILBRON KAMM and MORTON[27] and POHL[28] proved that an impurity present in ordinary cholesterol and phytosterols is responsible for the antirachitic efficacy after irradiation

1927 POHL [29] WINDAUS and HESS[30] and ROSENHEIM and WEBSTER[31] concluded from physical chemical and biological studies that the impurity in sterols or the provitamin D is ergosterol or a sterol of similar constitution such as a hypothetical dehydro-cholesterol Ergosterol had first been isolated by BRACONNOT in 1811 and was rediscovered in 1889 by TANRET [32]

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[14] A F Hes and L J Unger *P oc Soc Exptl Biol Med* 18 298 (1921)
[15] T F Zucker A M Pappenheimer and M Barnett *Ibid* 19 167 (1922)
[16] E V McCollum N Simmonds P G Shipley and E A Park *Ibid* 18 275 (1921) *J Biol Chem* 50 5 (1922)
[17] E M Hume *Lancet* II 1318 (19?2)
[18] O Sheets and C Funk *Proc Soc Exptl Biol Med* 20 80 (1922)
[19] A F Hess and M G Gutman *J Am Med Assoc* 78 29 (192?)
[20] H Steenbock *Science* 60 2?4 (19?4) H Steenbock and A Black *J Biol Chem* 61 405 (1924) H Steenbock and M T Nelson *Ibid* 62 209 (19?4)
[21] A F Hess *Am J Diseases Children* 28 517 (1924) A F Hess and M Weinstock *J Biol Chem* 62 301 (19?4)
[22] A F Hess M Weinstock and F D Helman *Ibid* 63 305 (1925)
[23] H Steenbock and A Black *Ibid* 64 263 (1925)
[24] O Rosenheim and T A Webster *Lancet* I 10 5 (1925)
[25] F W Schlutz and M Morse *Am J Diseases Children* 30 199 (19 5)
[26] O Rosenheim and T A Webster *J Soc Chem Ind* 45 932 (19 6) *Biochem J* 21 127 (1927)
[27] I M Heilbron E D Kamm and R A Morton *J Soc Chem Ind* 45 93 (1926) *Biochem J* 21 78 (1927)
[28] R Pohl *Nachr Ges Wiss Göttingen Math phys k Klasse* III, 142 (1926)
[29] R Pohl *Ibid* III 185 (1927)
[30] A Windaus and A Hess *Ibid* III 175 (1927) A Windaus *Ibid* III 183 (1926)
[31] O Rosenheim and T A Webster *Lancet* I 306 (1927) *Biochem J* 21 389 (1927)
[32] The name ergosterol originates from ergot a black fungus which grows on the rye plant and from which ergosterol was first isolated C Tanret *Compt rend* 108 98 (1889) *Ann chim phys* (VI) 20 289 (1890) *Compt rend* 147 75 (1908) *Ann chim phys* (VIII) 15 313 (1908)

1929–1931 REERINK and VAN WIJK[33] isolated for the first time a crystallized vitamin D preparation made from activated ergosterol LINSERT[34] isolated the pure compound

1930 MUSSEHL and ACKERSON[35] and independently MASSENGALE and NUSSMEIER[36] showed that vitamin D obtained from ergosterol was not active for chicks when fed on the basis of Rat Units in amounts equal to those effective for chicks from cod liver oil

1933 WINDAUS and LANGER[37] prepared a new synthetic provitamin D 22 dihydro ergosterol from ergosterol

1934 BILLS MASSENGALE and IMBODEN[38] showed that the vitamin D of fish oils is not a single substance since tuna liver oil was less antirachitic than cod liver oil Rat Unit for Rat Unit in chickens

WADDELL[39] found that crude cholesterol after activation yielded a vitamin D which is as effective for chicks as cod liver oil fed in equivalent Rat Units Thus the provitamin present in cholesterol must be different from ergosterol

1935 WINDAUS LETTRÉ and SCHENCK[40] synthesized the *hypothetical natural provitamin D 7 dehydro cholesterol* from cholesterol

1936 BOER REERINK VAN WIJK and VAN NIEKERK[41] and later (1937) also WINDAUS and BOCK[42] isolated 7 dehydro-cholesterol from cholesterol (obtained from hog skin) BROCKMANN[43] SIMONS and ZUCKER[44] and HASLEWOOD and DRUMMOND[45] *isolated the vitamin D from tuna and from halibut liver oils in the form of* crystallized esters and proved that the vitamin itself is mainly or entirely activated 7 dehydro cholesterol (vitamin $D_3$)

1937 SCHENCK[46] obtained crystallized vitamin $D_3$ prepared by activation of 7 dehydro-cholesterol

1938 BILLS MASSENGALE HICKMAN and GRAY[47] isolated a new vitamin D of low biological activity by molecular distillation of cod liver oil

## THE CONCEPT OF PROVITAMINS D AND OF VITAMINS D

Compounds of the physiological efficacy of vitamins D occur only in the animal organism Plants contain materials which can be converted

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[33] E H Reerink and A van Wijk *Biochem J* 23 1294 (1929) 25 1001 (1931)

[34] O Linsert Annotation in *Ann* 489 269 (1931) A Windaus O Linsert A Lüttringhaus and G Weidlich *Ibid* 492 226 (1932)

[35] F E Mussehl and C W Ackerson *Poultry Sci* 9 334 (1930)

[36] O N Massengale and M Nussmeier *J Biol Chem* 87 423 (1930)

[37] A Windaus and R Langer *Ann* 508 105 (1933)

[38] C E Bills O N Massengale and M Imboden *Science* 80 596 (1934)

[39] J Waddell *J Biol Chem* 105 711 (1934)

[40] A Windaus H Lettré and F Schenck *Ann* 520 98 (1935)

[41] A G Boer E H Reerink A van Wijk and J van Niekerk *Proc Acad Sci Amsterdam* 39 622 (1936)

[42] A Windaus and F Bock *Z physiol Chem* 245 168 (1937)

[43] H Brockmann *Ibid* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)

[44] E J H Simons and T F Zucker *J Am Chem Soc* 58 2655 (1936)

[45] G A D Haslewood and J C Drummond *J Soc Chem Ind* 55 598 (1936)

[46] F Schenck *Naturwissenschaften* 25 159 (1937)

[47] C E Bills O N Massengale K C D Hickman and E L Gray *J Biol Chem* 126 241 (1938)

into vitamins D These are called "provitamins D There occur in nature a number of provitamins D and of vitamins D The vitamins D differ in their antirachitic effectiveness in various animals The number of known naturally occurring provitamins D and vitamins D is small the provitamins ergosterol and 7 dehydro cholesterol and the corresponding vitamins namely, vitamin $D_2$ which is also known as 'activated ergosterol' viosterol or calciferol and vitamin $D_3$ which is activated 7-dehydro cholesterol Two additional provitamins D have been claimed patentwise to occur in invertebrata but very little is known about them A number of other provitamins D and vitamins D have been prepared in the laboratory and it is suspected that some of these compounds may also occur in nature Furthermore the existence of two more naturally occurring vitamins D is indicated on the basis of their outstanding physiological properties

## PROVITAMINS D

A provitamin D is defined as a compound that can be activated to a vitamin D Provitamins D are compounds of the cyclo pentano perhydro phenanthrene skeleton and belong to the sterol family They are specifically characterized by a hydroxyl group in the 3 position and a system of conjugated double bonds in ring B of the steroid nucleus, namely, in the 5 6 and 7 8 positions Provitamins D cannot be defined physiologically but it is suspected that it will be shown eventually that those steroids of the above classification which after activation are potent vitamins D for a specific species are absorbed in the intestinal tract of an animal of that species

According to this definition several compounds have been tentatively classified as provitamins D They will be discussed in the following sections A number of other compounds which fulfill only partly the definition of provitamins as given above are discussed under Specificity of Vitamin D (page 406)

### 3 Occurrence

Provitamins D are widely distributed over the animal and plant kingdom While it is impossible to make definite statements as to what provitamin D occurs in various specific natural sources, certain generalizations can be made It appears that the most prevalent provitamin D in higher animals and in human beings is 7 dehydro-cholesterol Plants molds and

yeast contain predominantly ergosterol [48] Considerable uncertainty exists about the kind of provitamins D in lower animals Thus, ergosterol occurs in the snail *Arion empiricorium* and in the earthworm, and 7 dehydro cholesterol in the snail *Buccinum undatum* [49] Mussels[50] are said to contain a different provitamin D and the same has been claimed for periwinkles [51]

The provitamin D content in different sources varies considerably Thus in higher animals, the provitamin D content is the greatest in the skin, namely, about 4% of the total sterol content, whereas the sterols from the inner organs contain only from 0 1 to 0 5% provitamin D (This is due to the activation mechanism of provitamins D to vitamins D as will be explained later) The best sources of ergosterol are yeast and certain molds some of which contain this provitamin as practically the only sterol The highest concentration of 7 dehydro cholesterol has been found in a species of snails (*Buccinum undatum*) and is 27% of the total sterol fraction The following tables indicate, as far as is known, the content of ergosterol and of 7 dehydro cholesterol in the sterol fraction of various sources Since in most naturally occurring materials the type of provitamin is not known another table shows the general provitamin D content in sterols from various materials

TABLE I

ERGOSTEROL CONTENT OF VARIOUS MATERIALS

| Source | Provitamin D in sterols % |
|---|---|
| Dried yeast | 90–100 |
| Snail *Arion empiricorium* | 19–25 |
| Earthworm | 22 |
| Cottonseed oil | 5 |
| Scopolia root | 1 4 |

TABLE II

7 DEHYDRO CHOLESTEROL CONTENT OF VARIOUS MATERIALS

| Source | Provitamin D in sterols % |
|---|---|
| Pigskin | 3–6 |
| Snail *Buccinum undatum* | 17–27 |

[48] C Tanret *Compt rend* 108 98 (1889) *Ann chim phys* (VI) 20 289 (1890) *Compt rend* 147 75 (1908) *Ann chim phys* (VIII) 15 313 (1908)

[49] A Windaus *Nachr Ges Wiss Göttingen Math physik Klasse* III 185 (1936) F Bock and F Wetter *Z physiol Chem* 256 33 (1938)

[50] A G Boer J van Niekerk E H Reerink and A van Wijk *U S P 2 163 659*

[51] A. G Boer J van Niekerk E H Reerink and A van Wijk U S P 2 216 719

TABLE III

PROVITAMIN D CONTENT OF VARIOUS MATERIALS

| Source | Provitamin D in sterols % |
|---|---|
| Vertebrata | |
| Skin from man | 0 15–0 43 |
| cattle | 0 18 |
| calf | 0 68 |
| mice | 0 87 |
| chicken feet[51a] | 1 0–4 0 |
| chicken trunk[51] | 0 001–0 01 |
| Blood serum (cow) | 0 15 |
| Brain (cow) | 0 01 |
| Lung (calf) | 0 025 |
| Heart (calf) | 0 032 |
| Spleen (cow) | 0 045 |
| Placenta (cow) | 0 18 |
| Pancreas (cattle) | 0 18 |
| Invertebrata | |
| Lugworm (*Arenicola marina*) | 4–12 |
| Mussels (*Mytilus edulis*) | 9–10 |
| Oysters | 5–6 |
| Leech | 4 |
| Crabs (*Cancer pagurus*) | 0 32 |
| Sea anemones | 2–10 |
| Plants | |
| Cocksfoot grass | 0 80 |
| Rye grass | 1 5 |
| Wheat germ oil | 0 8 |
| Seaweed | 0 008 |
| Cabbage | 0 05 |
| Spinach | 1 0 |
| String beans | 0 1 |

## 4 Isolation

The isolation of provitamins D from natural sources involves two different steps namely the isolation of the total sterols and the separation of the provitamins from other sterols present The isolation of the total sterols is usually a simple process and consists in either first extracting

[51] H R. Rosenberg unpublished data.

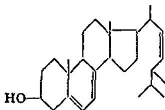
the total fat, followed by saponification of the fatty material and isolation of the unsaponifiable fraction, or in saponifying directly the total material followed by isolation of the non saponifiable fraction The isolation of the sterols from the non saponifiable fraction is usually carried out by crystallization from a suitable solvent, such as alcohol Special methods have been recommended for special cases For example, after saponification, the fatty acids may be precipitated as calcium salts which adsorb the sterols These are then recovered by solvent extraction from the filtered precipitate Another modification has been used in those cases where the total amount of fatty acids is very low Sodium benzoate is added to the saponification mass and the entire mixture is acidified Benzoic acid precipitates and adsorbs the sterols present which can then easily be isolated by alkaline extraction of the benzoic acid

The separation of the provitamins from other sterols is usually a difficult problem and success depends largely upon the type and amount of provitamin D present Thus no method has been found by which the provitamin D present in cholesterol from the spinal cord of cattle can be isolated satisfactorily, since the provitamin D content is only 0 1% Better chances for a successful isolation exist when the provitamin D is present in a concentration of at least 4–5% or more The usual method is fractional adsorption of the sterols or of their esters, for example, on aluminum oxide, which in many cases permits an almost quantitative separation If these methods fail, a condensation product with maleic or citraconic acid anhydride may be formed which can be split by thermal decomposition into the provitamin D and the acid anhydride

## 5 Properties

The following provitamins D are known

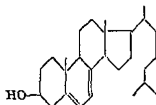
(1) Ergosterol Ergosterol crystallizes in small colorless crystals with water of crystallization The melting point varies according to the degree of hydration The best crystallized preparation contains $1^1/_2$ mols of water and melts at 168 C [11] Complete dehydration is very difficult to achieve and results in a product with a melting range from 166 to 183° C Ergosterol distills in high vacuum at 250° C without decomposition $[\alpha]_D^{20} = -130$ ($-135°$) and $[\alpha]_{5461}^{20} = -171°$ (in chloroform)



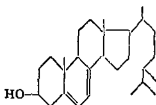

(2) Epi-ergosterol Physical constants unknown

[11] C E Bills and E M Honeywell *J Biol Chem* 80 15 (1928)

(3) 7-Dehydro-cholesterol M p 150–151° C [53] $[\alpha]_D^{20}$ = −113 6 in chloroform




(4) Epi 7 dehydro-cholesterol [54] M p 124–126° C $[\alpha]_D^{20}$ = −70 5 in chloroform
(5) 22 Dihydro-ergosterol [55] M p 152–153 C $[\alpha]_D^{20}$ = −109 in chloroform




(6) 22 23 Oxido ergosterol [56] Physical constants unknown
(7) "Mussel provitamin" [57] Constitution unknown M p 150–151 C $[\alpha]_D^{20}$ = −118° in benzene
(8) "Periwinkle provitamin" [58] Constitution unknown M p 137–137 5 C $[\alpha]_D^{20}$ = −124° in benzene
(9) 7 Dehydro-sitosterol M p 144–145 C $[\alpha]_D^{20}$ = −116 in chloroform
(10) 7 Dehydro-stigmasterol M p 154° C $[\alpha]_D^{20}$ = −113 15 in benzene

All these provitamins have similar solubility characteristics They are insoluble in water but soluble in the typical organic solvents, such as ether hydrocarbons chlorinated hydrocarbons alcohols etc The lower alcohols are usually used for recrystallization The provitamins separate from the alcohols with water (or solvent) of crystallization

All provitamins have the same characteristic absorption spectrum in *the ultraviolet which is characterized by maxima at 260, 270 281 and 293 5* mμ (Fig 15) The molecular absorption coefficient K = about 30 × 10³ for the band at 281 mμ

[53] A G Boer E H Reerink A van Wijk and J van Niekerk *Proc Acad Sci Amsterdam* 39 622 (1936)
[54] A Windaus and J Naggatz *Ann* 542 204 (1939)
[55] A Windaus and R Langer *Ibid* 508 105 (1933)
[56] A Windaus Linsal and K Buchholz quoted by K Dimroth and J Paland *Ber* 72 187 (1939)
[57] A G Boer J van Niekerk E H Reerink and A van Wijk U S P 2 163 659
[58] A. G Boer J van Niekerk E H Reerink and A van Wijk U S P 2,216 719

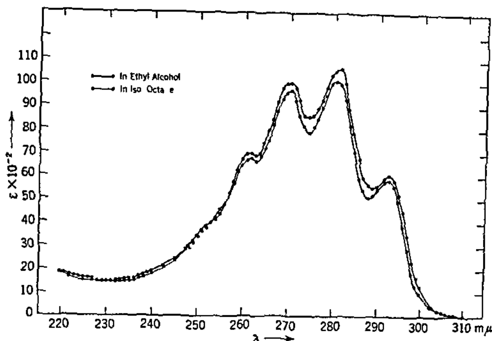



Fig 15—Absorption spectrum of ergosterol in ethanol and isooctane (T R Hogness A E Sidwell and F P Zscheile)

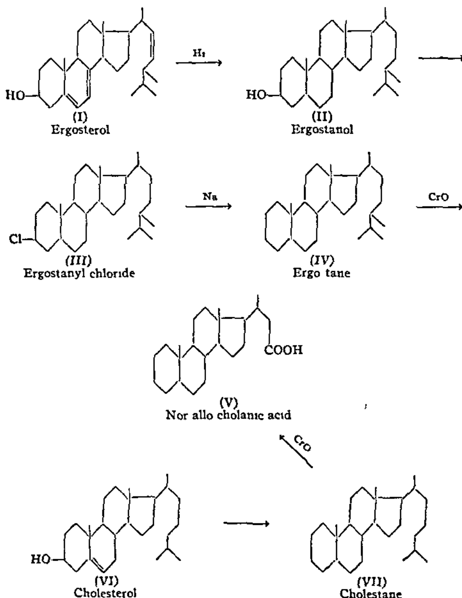
## 6 Chemical Constitution

### (a) *Ergosterol*

Ergosterol (I) has the empirical formula $C_{28}H_{44}O$ [59] which was established by careful analysis of derivatives containing hetero atoms, such as the 3 5 dinitro benzoate halogeno nitro benzoates etc The oxygen is present in a hydroxyl group, since esters can be obtained with acid anhydrides or acid chlorides in the presence of an amine Ergosterol contains three double bonds, since upon catalytic hydrogenation six atoms of hydrogen are absorbed [60] The totally saturated compound is called ergostanol (II) and has the formula $C_{28}H_{50}O$ It follows that four ring systems are present From ergostanol the corresponding hydrocarbon ergostane (IV) can be obtained by conversion into ergostanyl chloride (III) followed by reduction with sodium and amyl alcohol [61] Ergostane upon oxidation with chromic acid, yields a mono carboxylic acid, $C_{23}H_{38}O_2$ [62] which is identical with the nor allo cholanic acid (V) obtained from cholesterol

[59] A Windaus and A Lüttringhaus *Nachr Ges Wiss Göttingen Math physik Klasse* III 4 (1932) A Windaus F v Werder and B Gschaider *Ber* 65 1006 (1932)
[60] A Windaus and O Linsert *Ann* 465 154 (1928)
[61] F Reindel and E Walter *Ibid* 460 222 (1928)
[62] C K Chuang *Ibid* 500 270 (1933)

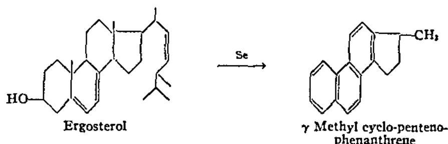
(VI) *via* cholestane (VII) This degradation reaction proves that ergos terol belongs to the class of sterols, which are characterized by the cyclo pentano perhydro phenanthrene skeleton Furthermore it follows that the steric configuration of cholestane and that of ergostane are the same




(I) Ergosterol
(II) Ergostanol
(III) Ergostanyl chloride
(IV) Ergo tane
(V) Nor allo cholanic acid
(VI) Cholesterol
(VII) Cholestane

That ergosterol belongs to the sterols has furthermore been demon strated since it yields upon total dehydrogenation with selenium

γ methyl cyclo penteno phenanthrene [63] which is the typical dehydrogenation product of all sterols No other class of compounds yields this particular hydrocarbon upon dehydrogenation




Ergosterol

γ Methyl cyclo-penteno-phenanthrene

The position of the hydroxyl group in the ring system was shown to be at carbon atom 3 [64] since acetylated ergostanol (VIII) yielded upon chromic acid oxidation, 3 acetoxy nor allo cholanic acid (IX) which was also obtained from dihydro cholesterol acetate (X) This result also proves that the steric configuration of the hydroxyl group is of the β type which was suspected since ergosterol can be precipitated with digitonin

$CH_3COO$ — $CrO_3$ → $CH_3COO$ … COOH

(VIII)
Ergostanol acetate

(IX)
3 Acetoxy nor allo-cholanic acid

↗ $CrO_3$

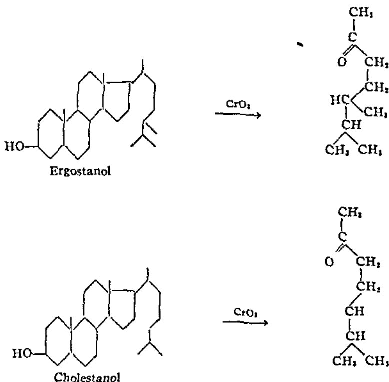
$CH_3COO$

(X)
Dihydro-cholesterol acetate

[63] O Diels and A Karstens *Ann* 478 129 (1930)

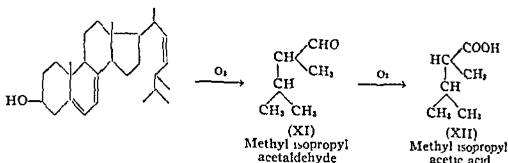
[64] E Fernholz and P N Chakravorty *Ber* 67 2021 (1934)

The chromic acid oxidations indicate furthermore, that the basic structure of the ring system and of the first five carbon atoms of the side chain is identical in both cholesterol and ergosterol Since ergosterol contains one carbon atom more than cholesterol this must be located in that part of the side chain which is removed during the oxidation This can be proved, since by energetic oxidation of ergostanol a ketone containing nine carbons ($C_9H_{18}O$) is obtained,[65] whereas cholestanol under the same conditions yields a ketone of only eight carbon atoms




Ergosterol upon ozonization yields methyl isopropyl acetaldehyde[66, 67] (XI) which was identified by the formation of its semicarbazone and dinitro phenyl hydrazone and by oxidation to methyl isopropyl acetic acid (XII) This result indicates that a double bond is in the side chain between carbon atoms 22 and 23

[65] A Guiteras Ann 494 116 (1932)
[66] F Reindel and H Kipphan Ibid 493 181 (1932)
[67] A Guiteras Ibid 494 116 (193 )

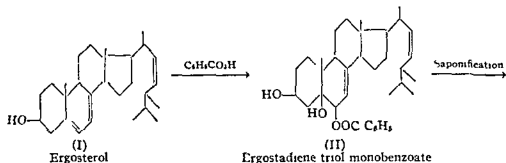


(XI)
Methyl isopropyl acetaldehyde

(XII)
Methyl isopropyl acetic acid

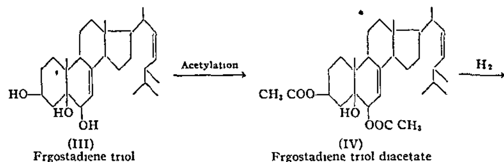
Another double bond is present in the 5 6 position which is the position of the double linkage in cholesterol This has been proved by the following series of reactions [68] Ergosterol (I) upon addition of perbenzoic acid to one of its double bonds yields an ergostadiene triol monobenzoate (II)[69] which contains one free secondary and one free tertiary hydroxyl group since only one of these can be readily esterified By saponification of this product followed by acetylation an ergostadiene triol diacetate (IV) is thus formed The diacetate upon catalytic hydrogenation takes up only one mol of hydrogen (forming V) and leaving one other double bond in the molecule This is rearranged by the action of hydrochloric acid The newly formed compound (VI) takes up hydrogen easily,[70] yielding the totally hydrogenated ergostane triol diacetate (VII) which upon saponification gives the free ergostane triol (VIII) Two of the hydroxyl groups in this compound are in $\alpha$ $\beta$ position to each other since upon oxidation with lead tetra acetate according to Criegée one atom of oxygen is consumed By chromic acid oxidation of the triol a hydroxy diketone (IX) is formed which splits out water by the action of hydrochloric acid The unsaturated diketone ergostene dione (X), can be transformed into a saturated diketone ergostadione (XI), by means of zinc and acetic acid Hydrazine condenses readily with the diketone with the formation of a pyridazine derivative (XII) The hydroxyl group originally present in ergosterol is in 3 position and therefore one of the keto groups in ergostadione is in 3 position Since the other keto group according to its reactions must be three carbon atoms removed from the keto group at carbon atom 3, it can only be located at carbon atom 6 The diketone is, therefore ergostadione 3 6 It follows furthermore, that the tertiary hydroxyl group in the ergostane triol is in 5 position the triol thus having the constitution of a 3,5 6 triol

[68] A Windaus H H Inhoffen and S v Reichel *Ann* 510 248 (1934)
[69] A Windaus and A Luttringhaus *Ibid* 481 127 (1930)
[70] I M Heilbron A L Morrison and J C E Simpson *J Chem Soc* 1933 302



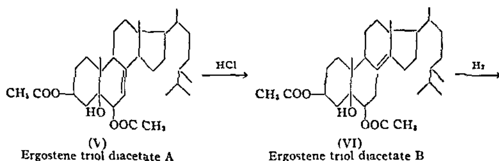

(I) Ergosterol

(II) Ergostadiene triol monobenzoate



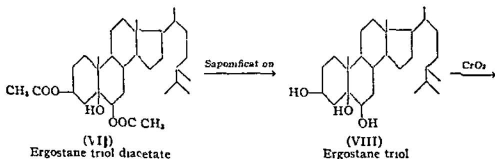

(III) Ergostadiene triol

(IV) Ergostadiene triol diacetate




(V) Ergostene triol diacetate A

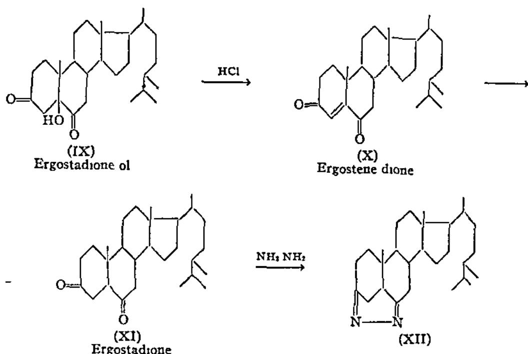
(VI) Ergostene triol diacetate B




(VII) Ergostane triol diacetate

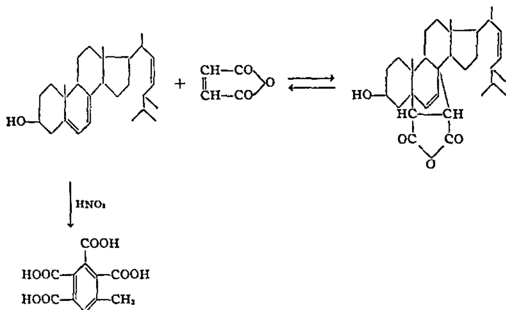
(VIII) Ergostane triol

(*Formula continued on following page.*)




The position of the third double bond is established by the refractive index [71] by x ray measurements,[72] and by the ultraviolet absorption spectrum all of which exhibit characteristics of a system of two conjugated double bonds The last double linkage cannot be located in the side chain due to the degradation reactions discussed above but must be in conjugation to the double bond in the 5,6 position Thus the only possible position is the 7,8 position The conjugation of the double bonds is furthermore suspected by the fact that ergosterol can be reduced with sodium and amyl alcohol[73] and by the fact that ergosterol forms a characteristic addition product with maleic anhydride[74] from which ergosterol can be recovered by thermal decomposition [75] Since it is known that one ring double bond is in the 5,6 position, this maleic anhydride condensation is only possible when the other double bond conjugated with the one in the 5 6 position is located in the same ring in which the first double bond is located Further proof is indicated by nitric acid oxidation of ergosterol which yields toluene 2,3 4 5 tetracarboxylic acid [6] While the value of this reaction by itself is limited since a migration of a methyl group is

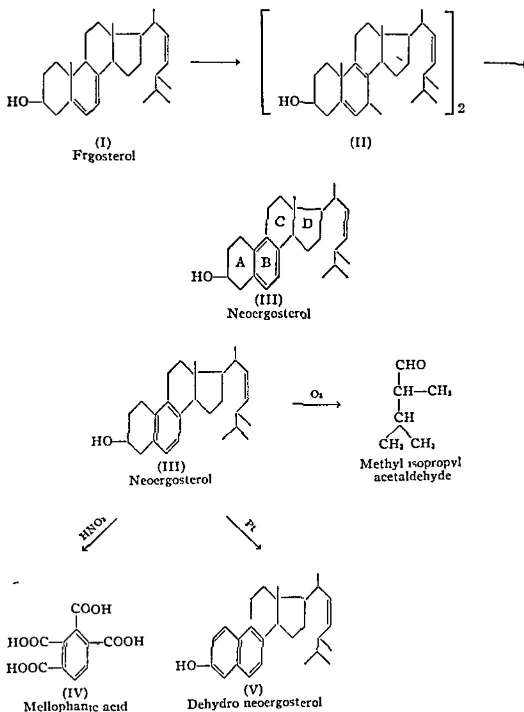
[71] K v Auwers and E Wolter *Nachr Ges Wiss Göttingen Math physik Klasse* III 101 (1931)
[72] G E R Schulze *Z physik Chem* A171 436 (1934)
[73] A Windaus and J Brunken *Ann* 460 225 (1928)
[74] A Windaus and A Luttringhaus *Ber* 64 850 (1931)
[75] H H Inhoffen *Ann* 508 81 (1933)
[76] F Reindel and K Niederländer *Ibid* 482 264 (1930) H H Inhoffen *Ibid* 494 122 (1932)




involved the result is corroborated by another series of reactions Ergosterol is dehydrogenated by eosin in the absence of oxygen when exposed to visible light with the formation of a bimolecular compound,[77] in which two molecules are linked together through the 7 7′ position with a shift of the 7 8 double bond into the 8 9 position[78] (II) Upon thermal de composition of this compound methane is evolved[79] and neoergosterol (III) is formed [77, 80] which contains an aromatic ring as indicated by the absorption spectrum and by the fact that only one double bond can be detected by perbenzoic acid or by catalytic hydrogenation That this double bond is in the side chain is proved by ozonization of neoergosterol which yields methyl isopropyl acetaldehyde [81] The reaction of nitric acid on neoergosterol yields mellophanic acid (benzene 1 2 3,4 tetracar boxylic acid (IV)) which differs from the nitric acid oxidation product of ergosterol by the absence of a methyl group While these results can be explained only by the assumption that ring B is aromatic, further proof has been brought forward The hydroxyl group of neoergosterol is ali cyclic in character, not phenolic thus excluding the possibility that ring A has become aromatic On the other hand, upon catalytic dehydrogena tion with platinum a β naphthol derivative, dehydro neoergosterol (V), is

[77] A Windaus and P Borgeaud *Ann* 460 235 (1928)
[78] H H Inhoffen *Naturwissenschaften* 25 125 (1937)
[79] H H Inhoffen *Ann* 497 130 (193 )
[80] K. Bonsted *Z physiol Chem* 185 165 (1929)
[81] H H Inhoffen *Ann* 497 130 (1932)

obtained[8] This result excludes the possibility that ring C in neoergosterol is aromatic in character




(I) Ergosterol

(II)

(III) Neoergosterol

(III) Neoergosterol

Methyl isopropyl acetaldehyde

(IV) Mellophanic acid

(V) Dehydro neoergosterol

[8] H Honigmann *Ann* 511 292 (1934)

Ergosterol is a very sensitive compound Acids cause rearrangements of the double bonds oxygen brings about the formation of peroxides and hydrogen causes the formation of a number of different di and polyhydro compounds

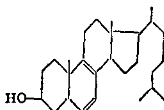
## (b) *The Other Provitamins D*

The proof for the chemical constitution of the other provitamins D is mainly based upon the determination of the characteristic absorption spectrum which is common to all provitamins D since they all have the same system of conjugated double bonds in ring B The constitution of the provitamins 7 dehydro cholesterol and 22 dihydro ergosterol is further more established since these compounds have been obtained by synthetic methods from cholesterol and from ergosterol respectively The constitution of the epi compounds namely of epi ergosterol and of epi 7 dehydro cholesterol is established since these provitamins do not precipitate with digitonin whereas the parent provitamins ergosterol and 7 dehydro cholesterol form addition compounds with digitonin This reaction in combination with their absorption spectrum and the method of synthesis of the epi compounds proves that they differ from their parent provitamins D only in the stereochemical configuration of the hydroxyl group in 3 position This position of the 3 hydroxyl group must be related to some other group in the sterol ring system Ruzicka chose the relationship between the substituents on $C_3$ and on $C_5$ as reference for the *cis trans* isomerism of the 3 hydroxyl group Since the provitamins D have no hydrogen at $C_5$ this system applies by implication Ergosterol 7 dehydro cholesterol etc belong according to this nomenclature to the *trans* derivatives whereas the compounds of the epi family which do not precipitate with digitonin belong to the *cis* derivatives Another system of nomenclature suggested by Schoenheimer uses the relation of the hydroxyl group in 3 position to the methyl group on carbon atom 10 According to this system ergosterol and 7 dehydro cholesterol belong to the *cis* $C_3$ $C_{10}$ compounds and the epi derivatives belong to the *trans* $C_3$ $C_{10}$ compounds

The constitution of the mussel provitamin D and of the periwinkle provitamin D are unknown It has tentatively been assumed that the mussel provitamin D has 29 carbon atoms[43] that is it has one carbon atom more than ergosterol or two carbon atoms more than 7 dehydro cholesterol

[43] A. G Boer J van Niekerk E H Reerink and A van Wijk U S P 2 163 659

benzoates[94] or of substituted benzoates[95] Isodehydro cholesterol has the probable formula (VIII),[95] that is, it differs from 7 dehydro cholesterol only in the position of the double bonds, which are believed to be in the 6 7 and 8,9 positions




(VIII)
Isodehydro-cholesterol

Besides this clear cut synthesis a number of other methods have been found to yield 7 dehydro cholesterol in small amounts Thus by direct oxidation of cholesterol under mild conditions[96] for example, with per oxides, a provitamin D is obtained which is probably 7 dehydro cholesterol Somewhat higher yields are apparently obtained by the utilization of quinones as dehydrogenating agents[97 98] Also methylene blue in the presence of light, and succino dehydrogenase have been claimed to convert cholesterol into provitamin D[99]

A number of other provitamins D are claimed to have been obtained from cholesterol or its derivatives The following methods used for their preparation indicate that the provitamins obtained may ultimately prove to be 7 dehydro cholesterol, although some investigators believe them to be new and different provitamins D[100]

(*a*) Cholesterol upon heating, is converted in very small amounts into a provitamin D[101]

(*b*) Cholesterol freed essentially from its naturally adherent pro vitamin D content and subjected to ultraviolet light, yields a vitamin D[102]

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[94] A G Boer E H Reerink A van Wijk and J van Niekerk *Proc Acad Sci Amsterdam* 39 622 (1936)

[95] A Windaus O Linsert and H J Eckhardt *Ann* 534 22 (1938)

[96] J Waddell U S P 2 028 364 U S P 2 056 992

[97] N A Milas and R Heggie *J Am Chem Soc* 60 984 (1938)

[98] P I T Sah *Rec t av chim* 59 454 (1940)

[99] N A Milas and R Heggie *J Am Chem Soc* 60 984 (1938)

[100] C E Bills *Cold Spring Harbor Symposia Quant Biol* 3 328 (1935)

[101] E M Koch and F C Koch *Science* 82 394 (1935) *J Biol Chem* 116 756 (1936) M L Hathaway and D E Lobb *Ibid* 113 105 (1936) R W Hamann and H Steenbock *Ibid* 114 505 (1936)

[102] C E Bills *J Biol Chem* 66 451 (1925) A Jendrassik and A G Keményffi *Biochem Z* 189 180 (1927) S K Kon F Daniels and H Steenbock *J Am Chem Soc* 50 2573 (1928) F C Koch E M Koch and J K Ragins *J Biol Chem* 85 141 (1929) E M Koch and H B Lemon *Ibid* 85, 159 (1929)

This vitamin D is alleged to be different from that obtained from 7 dehydro cholesterol since it was found to be less effective for chicks

(c) 7 Hydroxy cholesterol upon irradiation develops a slight anti rachitic potency[103] The work does not indicate whether or not pure 7-hydroxy cholesterol was employed There exists the possibility that 7 hydroxy cholesterol can lose one mol of water to form 7 dehydro cholesterol

(d) 7 Oxo cholesterol acetate upon Grignard reaction with isobutyl magnesium bromide followed by heating to 200° C is said[104] to produce small amounts of a new provitamin D While this statement has not been proved, it appears possible that the Grignard compound led to a partial reduction to a 7 hydroxy cholesterol compound Such reductions have been observed to occur in sterols[105] 7 Hydroxy cholesterol may then upon heating be transformed partially into 7 dehydro cholesterol

### (b) *The Synthesis of Epi 7-dehydro cholesterol*

The synthesis of epi 7 dehydro cholesterol has been carried out (1) by using epi cholesterol as starting material[106] and employing the methods outlined for the synthesis of 7 dehydro cholesterol and (2) by epimerization of the 3 hydroxyl group of 7 dehydro cholesterol[107] The latter compound upon oxidation with aluminum *tert* butyroxide yields a dehydro cholestenone which upon reduction with aluminum isopropoxide is converted into epi 7 dehydro cholesterol in a yield of 1 25%

### (c) *The Synthesis of Epi ergosterol*

Epi ergosterol has been obtained in about 1 3% yield by reduction of ergosterone with aluminum isopropoxide but has not been isolated in a high state of purity[108]

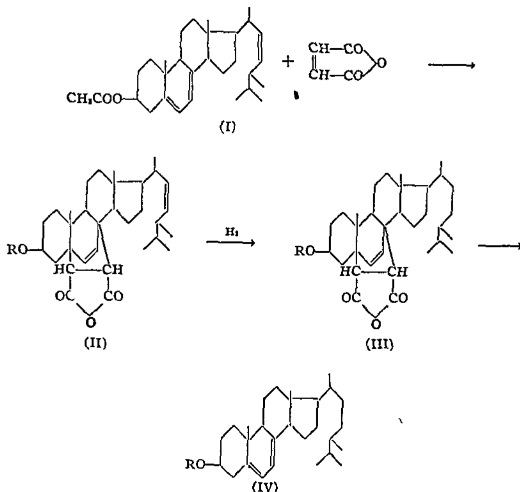
### (d) *The Synthesis of 22 Dihydro ergosterol*

22 Dihydro ergosterol has been obtained by side chain hydrogenation of ergosterol[109] This is carried out by acetylating ergosterol followed by formation of an addition product of maleic anhydride with ergosterol

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[103] C. E Bills *Cold Spring Harbor Symposia Quant Biol* 3 328 (1935)
[104] S Weinhouse and M S Kharasch *J Org Chem* 1 490 (1936)
[105] L Ruzicka and H R Rosenberg *Helv Chim Acta* 19 357 (1936)
[106] A Windaus and J Naggatz *Ann* 542 204 (1939)
[107] A Windaus and O Kaufmann *Ibid* 542 218 (1939)
[108] A Windaus and K Buchholz *Ber* 71 576 (1938) 72 597 (1939)
[109] A Windaus and R. Langer *Ann* 508, 105 (1933)

acetate (II) This, upon catalytic hydrogenation, is selectively reduced in the side chain (III) By thermal decomposition, 22 dihydro ergosterol acetate (IV) is obtained This yields 22 dihydro ergosterol upon saponification




*(e) Synthesis of 22 23 Oxido ergosterol*

22 23 Oxido ergosterol has been mentioned in the literature[110] but the method of synthesis has not been reported as yet Probably the maleic anhydride addition product of an ergosterol ester is treated with a mild oxidizing agent to form the 22 23 oxido compound which upon thermal decomposition yields the 22 23 oxido ergosterol ester

110 A Windaus, Linsel and K Buchholz quoted by K Dimroth and J Paland *Ber* 72 187 (1939)

### (*f*) *Synthesis of 7 Dehydro sitosterol*

The synthesis of 7 dehydro sitosterol has been carried out[111] according to the Windaus method for the synthesis of 7 dehydro cholesterol As starting material the sitosterol mixture from soybean oil was used This contains various isomers which are extremely difficult to separate

### (*g*) *Synthesis of 7 Dehydro stigmasterol*

7 Dehydro stigmasterol has been synthesized[112] from stigmasterol according to the previously outlined method for the synthesis of 7 dehydro cholesterol from cholesterol

## 8 Industrial Methods of Preparation

The industrial preparation of the provitamins ergosterol and 7 dehydro cholesterol is of technical importance The provitamin D from certain marine animals is also used commercially Ergosterol is manufactured mainly by extraction from yeast and to a small extent also from the mycelium of aspergillus niger Commercial ergosterol contains as much as 5% of α dihydro ergosterol[113] which accompanies ergosterol in fungi and which has about the same solubility characteristics as ergosterol Extensive investigations on the ergosterol content of yeast revealed that the yields are more dependent upon the conditions under which the yeast is grown than upon the strain of yeast

7 Dehydro cholesterol is synthesized from cholesterol according to the methods described previously

## 9 Biogenesis

Little is known about the biogenesis of the provitamins D Ergosterol is synthesized by yeast and by a number of fungi and other lower organisms This synthesis is apparently more closely related to the metabolism of carbohydrates than to that of fats[114] On the other hand aspergillus niger can synthesize ergosterol when sodium acetate is the sole source of carbon[115, 116] No connection could be demonstrated between sterol

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[111] W Wunderlich *Z phys ol Chem* 241 116 (1936)
[112] O Linsert *Ibid* 241 125 (1936)
[113] R K. Callow *Biochem J* 31 87 (1931)
[114] C E Bills *Physiol Rev* 15 1 (1935) A Heidıschka and H L dner *Z physiol Chem* 181 15 (1929)
[115] W H Maguigan and E Walker *B och m J* 34 804 (1940)
[116] I Smedley MacLean and D Hoffert *Ib d* 17 7 0 (19?3) R So de hoff a d H Thomas *Ann* 530 195 (1937)

synthesis and the nitrogen metabolism [117] [118] 7 Dehydro cholesterol is apparently synthesized by higher animals and by human beings The course of this synthesis is not known, but it is perhaps significant that both forms of 7 hydroxy cholesterol the $\alpha$ and the $\beta$ isomer, apparently occur in animal tissues Thus the formation of 7 dehydro cholesterol by dehydration of the 7 hydroxyl compounds appears possible It has also been postulated that 7 dehydro cholesterol is synthesized from cholesterol by enzymatic dehydrogenation No proof for this hypothesis can be offered other than the observation that small amounts of 7 dehydro cholesterol and of dihydro cholesterol always are present with cholesterol [119] It has been predicated therefore that while one molecule of cholesterol is dehydrogenated another molecule is hydrogenated On the other hard, it seems plausible to assume that in certain cells 7 dehydro cholesterol is totally synthesized in a manner similar to the total synthesis of cholesterol which has been reported as being built up from compounds of only two or three carbon atoms [120]

## 10 Determination

### (a) *Physical Methods*

The most accurate method for the determination of provitamins D is the spectroscopical analysis The characteristic absorption spectrum of the provitamins in the ultraviolet region is the same for all provitamins on a molecular basis This method while quite accurate for the determination of provitamins D in sterol mixtures isolated from natural sources cannot distinguish between the individual provitamins D This method can also be used for the determination of provitamins D in preparations obtained by chemical synthesis with the restriction that the actual absorption maxima must be determined as such and in relation to each other and that general absorption must be observed and properly considered In dubious cases, the addition of known amounts of a provitamin D may increase the accuracy of the determination of the original provitamin D content

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[117] W H Maguigan and E Walker *Biochem J* 34 804 (1940)

[118] O N Massengale C E Bills and P S Prickett *J Biol Chem* 94 213 (1931) M Sobotka W Halden and F Bilger *Z physiol Chem* 234 1 (1935) F Reindel K Niederländer and R Pfundt *Biochem Z* 291 1 (1937)

[119] R Schönheimer H v Behring R Hummel and L Schindel *Z physiol Chem* 192 73 (1930) *Naturwissenschaften* 18 156 (1930)

[120] D Rittenberg *J Biol Chem* 119 LXXXIII (1937)

### (b) *Chemical Methods*

No chemical method has been developed which is specific enough to determine the provitamins D accurately Since all methods known are based on the characteristic conjugated unsaturation of the provitamin D molecule no distinction can be made between the various provitamins The chemical color tests based on this unsaturation are the following

1 **The Reversed Salkowski Reaction**[121] The provitamin D is dissolved in chloroform and concentrated sulfuric acid is added The acid becomes deep red while the chloroform layer remains colorless Sterols such as cholesterol and sitosterol give the opposite color reactions the chloroform becomes red while the acid exhibits a green fluorescence

2 **The Liebermann-Burchard Reaction**[122] The provitamin D is dissolved in chloroform, and acetic anhydride and concentrated sulfuric acid are added dropwise A red color develops which progressively changes from blue violet to green Cholesterol gives a similar change of color but the initial red color remains unchanged for a longer period of time

3 **The Tortelli-Jaffe Reaction**[123] A solution of provitamin D in acetic acid is mixed with a 2% solution of bromine in chloroform A green color develops This reaction is given by steroids with a di tertiary ethenoid linkage[124] and by vitamins D

4 **The Rosenheim Reaction**[125] To a solution of provitamin D in chloroform a solution of trichloro acetic acid in water is added A red color develops which changes slowly into a light blue This color reaction becomes considerably more sensitive when prior to the addition of the tri chloro acetic acid, lead tetra acetate in glacial acetic acid is added to the chloroform solution of the provitamin An intense green fluorescence occurs which is however not given by provitamin D esters Thus, provitamins can be differentiated from their esters by this method This modified color reaction can be employed for the detection of provitamins D in an amount of the order of 0 1 $\gamma$[126]

5 **Chloralhydrate Reaction** Crystals of provitamin D, when heated slowly with crystals of chloralhydrate melt above 50° C and the

[121] E Gérard *Chem Zentr* (1895) II 229 *A ch ges Physiol Pflügers* 6 207 (1872)

[122] C Liebermann *Be* 18 1804 (1885) A He duschka and H L ndner *Z physiol Chem* 181 15 (19 9)

[123] E P Häussler and E Brauchli *Helv Chim Acta* 12 187 (1929) I M He lbron and F S Spri g *Biochem J* 24 133 (1929)

[124] U Westphal *Ber* 72 1243 (1939)

[125] O Rosenheim *Biochem J* 23 47 (1929) 25 74 (1931)

[126] A on Christiani and V Anger *Ber* 72 1124 1482 (1939)

mixture becomes first red, then green and finally deep blue  Sterols, such as cholesterol, do not give any color reaction with chloralhydrate

6  The Antimony-Trichloride Reactions [127]  Provitamin D dissolved in chloroform and mixed with a solution of antimony trichloride in chloroform yields a red color

7  The Tschugajeff Reaction [130]  A solution of provitamin D in glacial acetic acid with an excess of acetyl chloride and zinc chloride yields upon heating to the boiling point an eosin red color with a greenish yellow fluorescence  The *sensitivity is reported to be 1 80,000*

While none of these reactions is absolutely quantitative the Liebermann Burchard reaction and the Rosenheim reaction[128, 129] have been recommended and developed for quantitative assays

## CONVERSION OF PROVITAMINS D TO VITAMINS D

### 11  Process of Activation

Provitamins D can be activated to vitamins D by a number of different processes  *They all involve in principle the input of energy into the provitamin D molecule*  Thus ultraviolet light, cathode rays, radium emanation etc, effect activation

### (a) *Ultraviolet Light Activation*

Provitamins D are activated to vitamins D by ultraviolet light of the same wave lengths as those which are absorbed by the provitamins as evident from the absorption spectrum  The energy required to produce one U S Pharmacopoeia unit of vitamin D from ergosterol has repeatedly been investigated [131]  The data obtained *under the most careful conditions* indicate that 7 5 $\times 10^{13}$ quanta will produce one U S Pharmacopoeia unit of vitamin D [132]  In the active region, the energy necessary *depends* upon the wave length  The most effective activation of ergosterol is obtained from light of the wave length 281 m$\mu$, which is the line that shows maximum absorption of ergosterol  On the other hand the *activation*

[127] E P Haussler and E Brauchli *Helv Chim Acta* 12 187 (1929)  I M Heilbron and F S Spring *Biochem J* 24 133 (1929)

[128] R K Callow *Biochem J* 25 87 (1931)

[129] A von Christiani and V Anger *Ber* 72 1124 1482 (1939)

[130] L Tschugajeff *Chem Ztg* 24 542 (1900)  *Z angew Chem* 13 618 (1900)

[131] S Kon F Daniels and H Steenbock *J Am Chem Soc* 50 2573 (1928)  A L Marshall and A Knudson *Ibid* 52 2304 (1930)  T A Webster and R B Bourdillon *Biochem J* 22 1223 (1928)  R W Haman and H Steenbock *Ind Eng Chem Anal Ed* 8 291 (1936)

[132] R S Harris J W M Bunker and L M Mosher *J Am Chem Soc* 60 2579 (1938)

of 7 dehydro cholesterol has been reported[133] to be significantly greater by monochromatic light of 296 7 mμ than by any other wave length Con flicting results have been obtained from studies on the production of vitamin D in rats While in one laboratory light of 281 mμ was found most effective [134] in another laboratory a superior effectiveness of light of 296 7 mμ was found [135]

There are different types of ultraviolet light sources used for the activa tion of provitamins D namely the light of the magnesium arc and of the carbon arc and of the mercury vapor lamp The light emitted by a bismuth vapor lamp has also been recommended [136] Cored carbon elec trodes impregnated with various metals are also used

Provitamins D can be activated in the dry state [137] in vapor form[138] and in solutions Irradiation of provitamin D in the dry state gives poor yields because the vitamin D is produced only on the surfaces of the crystals and further irradiation destroys the vitamin D formed before the provitamin D present in the middle of the crystals has been affected Irradiation in the vapor phase has not been investigated thoroughly The best method of irradiation is to expose solutions of provitamins D to the action of the ultraviolet light Agitation of the solution[139] was found to enhance the vitamin D yield The best technical method known today involves the utilization of a special quartz irradiation chamber which is built concentrically around the mercury vapor lamp and through which the provitamin D solution passes continuously in turbulent flow [140]

As pointed out before the yield of vitamin D is influenced by the wave length of the light employed It is well established that light of wave length between 275 and 300 mμ produces the best yields of vitamin D[141] with the smallest amount of by products The desired light is obtained by special light filters Light below 275 mμ is filtered out by aromatic compounds such as benzene[142] or xylene[142] or by compounds such as di phenyl[143] in benzene solution, or by a 5% lead acetate solution [144] Car

[133] J W M Bunker R S Harris and L M Mosher *J Am Chem Soc* 62 508 (1940)
[134] A Knudson and F Benford *J Biol Chem* 124 287 (1938)
[135] J W M Bunker R S Harris and L M Mosher *J Am Chem Soc* 62 508 (1940)
[136] N V Philips Gloeilampenfabrieken Holland Dutch P 35 079 U S P 1 904 751
[137] H H Beard R B Burk H E Thompson and H Goldblatt *J Biol Chem* 96 307 (1932)
[138] F A Askew R B Bourdillon and T A Webster *Biochem J* 26 814 (1932)
[139] A Windaus K Westphal F v Werder and O Rygh *Nachr Ges Wiss Göttingen Math physik Klasse* III 45 (1929)
[140] F Seitz *Darstellung von Vitaminpräparaten* Leipzig 1939 50
[141] E H Reerink and A van Wijk *Strahlentherapie* 40 728 (1931) T H Rider G Sperti G P Goode and H G Cassidy *J Am Med Assoc* 106 452 (1936)
[142] N V Philips Gloeilampenfabrieken G P 634 146
[143] I G Farbenindustrie G P 565 900
[144] General Development Lab Inc U S P 1 982 029

bon tetrachloride is used to filter out light of the wave lengths 312 and 313 mμ [145] Special types of glass which selectively allow light of 270–300 mμ to penetrate can also be made

The presence of oxygen during the irradiation should be carefully avoided [146] The intermediates and by products formed during the irradiation are much more susceptible to oxidation by molecular oxygen than either the provitamins or the vitamins D The presence of oxygenated materials makes it difficult to isolate crystalline vitamin D, but the actual yield is not essentially affected [147]

*There is apparently also a specific solvent effect involved in the irradia*tion procedure The activation takes place more rapidly in ether than in alcohol [148]

Other materials such as cyclohexane and the diether, dioxane have also been recommended[149] as solvents either alone or in mixture with ethyl acetate benzene or triethanolamine Provitamins D can also be irradiated in oil solution Of further interest are some special methods of irradiation Thus it is claimed that enhanced yields of vitamin D are obtained when compounds are added which protect the vitamin D after its formation Ethylene or alkalies have been used in this manner [150] The irradiation can also be carried out in the presence of photosensitizers, for example of eosin erythrosin or dibromo dinitro fluorescein [151]

The temperature coefficient of activation, if it exists, is very small [152] The enhanced effect observed when irradiating at the boiling point of the solvent[153] is probably due to more uniform activation of all molecules present

The best yields are obtained when only 40 to 60% of the total provitamin D is converted [154] *In such cases, the yields are between 30%* and 60% of theory This calculation includes the recovery of unchanged provitamin D

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[145] N V Philips Gloeilampenfabrieken Holland B P 385 626

[146] A Smakula *Nachr Ges Wiss Göttingen Math physik Klasse* III 49 (1928) C E Bills E M Honeywell and W M Cox *J Biol Chem* 80 557 (1928) E H Reerink and A van Wijk *Biochem J* 23 1?94 (1929)

[147] T C Angus F A Askew R B Bourdillon H M Bruce R K Callow C Fischermann J S L Philpot and T A Webster *Proc Roy Soc (London)* B108 340 (1931)

[148] C E Bill E M Honeywell and W M Cox *J Biol Chem* 92 601 (1931)

[149] Standard Brands Inc U S P 1 955 5?4

[150] I G Farbenindustrie B P 321 992

[151] E Merck Co B P ?86 66?

[152] C E Bills and F G Brickwedde *Nature* 121 452 (1928) T A Webster and R B Bourdillon *Biochem J* 22 1223 (1928)

[153] I C Farbenindustrie U S P 1 896 191 Société Usines Chimiques du Rhône Poulenc B P 335 277

[154] N V Philips Gloeilampenfabrieken G P 634 146

Direct irradiation of foods has attracted special attention. Thus, cereals and flour have been commercially irradiated. Of considerable practical importance is the irradiation of milk which is carried out in special equipment since ultraviolet light penetrates milk only slightly and since the odor of milk is easily influenced by ultraviolet light. Yeast and dried milk are also irradiated commercially.

### (b) *Activation by Other Means*

Although activation by ultraviolet light has been the only method thoroughly investigated, a number of other methods are known for the conversion of provitamins D into vitamins D.

Cathode rays as such[155] or in the presence of catalysts[156] such as iron-uranium salts, canal rays,[157] α, β and γ rays of radioactive elements,[157] radium emanation,[158] x rays,[157, 1 9] corpuscular rays, electrons of high frequency[160] and finally alternating current of high frequency[161] have been claimed to effect activation of provitamins D. Most of these claims must be further investigated before they can be accepted. The conversion of provitamins to vitamins D by mitogenetic radiation[162] has also been postulated and will be discussed in the section on the Biogenesis of Vitamin D (page 404).

## 12 Mechanism of Activation

The conversion of provitamins D to vitamins D is not a simple process. During the course of this reaction several substances are formed[163, 164, 165] before the vitamin D is obtained and the latter is not stable to the activating energy but is transformed into other compounds.

The mechanism of the provitamin D conversion to vitamin D has been studied extensively in the case of ergosterol. The activating energy was

[155] A Knudson and C N Moore *J Biol Chem* 81 49 (1929) R M Hoffman and F Dan ls *Ibid* 115 119 (1936)

[156] Am rican Research Prod Inc U S P 1 983 944

[157] K Hembd and Vitam F brik G P 577 170

[158] R B Moore and T DeVries *J Am Chem Soc* 53 ?676 (1931)

[159] See however H Goldblatt *E geb Allg Path and Path Anat* 2 *Abt* 25 58 (1931)

[160] Brit Thomson Houston Co B P 292 9?6

[161] *I G Farb nindustri Austrian P 119 210*

[162] H M i *Abhandl aus der K nde heilk nde u ihren Grenzgeb* 1937 H 45

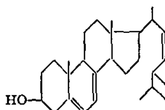
[163] C E Bills and F G Brickw dde *Nat e* 121 452 (1928) T A Webster and R B Bourd llon *B ochem J* 22 1223 (19 8)

[164] A Smakula *Nach Ges Wiss Götti g n Math physik Klasse* III 49 (19?8) C E B lls E M Honeywell and W M Cox *J Bi l Chem* 80 557 (19?8) E H Reer nk and A van Wijk, *Biochem J* 23 1?94 (19?9)

[165] O Rosenheim and T A Webster *Lancet* II 62? (1927)

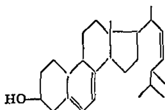
supplied in these studies by ultraviolet light It is not certain that the same intermediate products, vitamin D and final products are obtained by processes other than the ultraviolet irradiation It seems relatively certain, however, that at least the vitamin is identical from all forms of activation [166] The mechanism, which will be described for ergosterol in the following paragraphs, is believed to be identical for all provitamins D and certain indications are available for this assumption other than analogy, especially in the case of the activation of 7 dehydro cholesterol[167] and 22 dihydro ergosterol [168]

The photochemical process is irreversible, that is, there is no equilibrium between the irradiation products The number of the irradiation products and the sequence of these compounds during the course of the process were revealed by actual isolation of the pure intermediates and by a determination of all the products obtained from each isolated intermediate upon further irradiation The result of these investigations is pictured in the following scheme for the reaction mechanism [169]




Ergosterol, m p 166° C $[\alpha]_D^{20} = -132$ in chloroform Absorption maxima at 260 270 282 and 293 5 mμ






Lumisterol$_2$, m p 118° C $[\alpha]_D^{19} = +192°$ in acetone Absorption maxima at 265 and 280 mμ

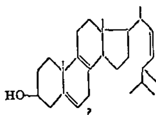


[166] For the vitamin made by activation of ergosterol by low velocity electrons see I McQuarrie W H Thompson A V Stoesser and L G Rigler *J Pediat* 10 295 (1937)

[167] A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)

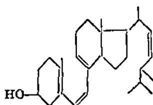
[168] A Windaus and B Guntzel *Ibid* 538 120 (1939)

[169] P Setz *Z physiol Chem* 215 183 (1933)



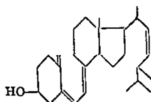
Pro-tachysterol$_2$



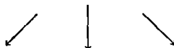


Tachysterol$_2$ $[\alpha]_D^{18} = -70$ in benzene Absorption maxima at 268 280 and 294 mμ





Vitamin $D_2$ (Calciferol) M p 116° C $[\alpha]_D^{20} = +106°$ in alcohol Absorption maximum at 265 mμ



Toxisterol$_2$ Absorption maximum at 248 mμ

Suprasterol$_2$ I M p 104 C $[\alpha]_D^{18} = -76$ in chloroform Absorption only in far ultra violet

Suprasterol$_2$ II M p 110 C $[\alpha]_D^{1} = +63$ in chloroform Absorption only in far ultraviolet

While there is no assurance that all intermediate products of the irradiation of ergosterol have been recognized, there is no evidence that other products are formed During irradiation, the ultraviolet absorption characteristics change with the compounds obtained This change is illustrated by the following curves of the irradiation products (Fig 16)

All reaction products of the above scheme are isomers of the provitamins

The constitutions of lumisterol, tachysterol and vitamin D are well established, the formula for pro tachysterol is hypothetical

The nomenclature of the irradiation products of the various provitamins D has been proposed by Windaus[170] *Accordingly, the intermediates will* be called "lumisterol," "pro tachysterol" "tachysterol "vitamin D, etc , and the special structure due to their derivation from specific provitamins D will be designated by small index numbers such as vitamin $D_1$ vitamin $D_2$ lumisterol$_3$," etc The index numbers for all irra-

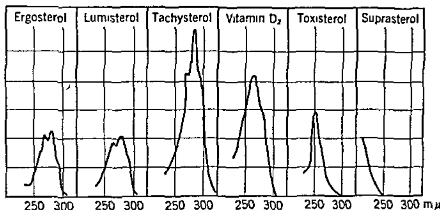



Fig 16 —Absorption curves of ergosterol and its irradiation products in 0 2% etherial solution (A Windaus)

diation products from one provitamin are the same, for example all products derived from ergosterol are called products$_2$, from 7 dehydro cholesterol, products$_3$ from 22 dihydro ergosterol, products$_4$ There are no products$_1$ The term vitamin $D_1$ was a misnomer given originally to the first isolated crystalline material of vitamin D activity[171] which later proved to be a molecular addition product of lumisterol$_2$ and vitamin $D_2$[172]

All photochemical reaction products formed upon irradiation of any given provitamin D are subject to further photochemical attack The irradiation can however, be directed so that one or the other product is obtained predominantly according to the wave length used for the reaction

Of all the irradiation products formed and isolated only the one which is called "vitamin D exerts antirachitic action The others are physiologically inert or exhibit physiological properties entirely different from those of the natural vitamins D

[170] A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)

[171] A Windaus *Proc Roy Soc (London) B108 568 (1931)* A Windaus A Luttringhaus and M Deppe *Ann* 489 252 (1931)

[172] A Windaus and A Luttringhaus *Z physiol Chem 203 70 (1931)* *A Windaus* O *Linsert* A Lüttringhaus and G Weidlich *Ann* 492 296 (1932)

## 13 Chemistry of Activation Products

(a) Lumisterol (Sterol X) The first irradiation product of ergosterol which can be detected is lumisterol$_2$ Lumisterol$_2$ can be prepared predominantly by irradiation of ergosterol with light of the wave length 290–300 mμ [173] Upon conversion of about 40% of the ergosterol used the unchanged ergosterol is separated by crystallization from methanol The mother liquors are evaporated to dryness and upon crystallization from acetone the molecular addition product of vitamin $D_2$ and lumisterol$_2$ which was called ' vitamin $D_1$ separates The addition product is broken up by acetylation, followed by fractional crystallization from acetic acid, whereby the lumisterol$_2$ acetate crystallizes first Lumisterol$_2$ itself is obtained by saponification of its acetate

Experimental investigations[174] have been made to ascertain if lumisterol$_2$ is a necessary intermediate or if ergosterol can be converted directly into the next intermediate tachysterol$_2$ From the changes involved in the absorption spectrum of ergosterol and of lumisterol$_2$ upon short irradiation with ultraviolet light it is concluded[175] that lumisterol$_2$ must be formed before tachysterol$_2$ can be obtained

Analysis and molecular weight determinations indicate that lumisterol$_2$ is an isomer of ergosterol The hydroxyl is present since esters can be formed Lumisterol$_2$ contains three double bonds as evident from titrations with perbenzoic acid and from catalytic hydrogenation [176] [177] One of these is in the side chain in 22 23 position as in ergosterol since upon ozonolysis methyl isopropyl acetaldehyde is obtained [178] The other two double linkages are conjugated as evident from the absorption spectrum (Fig 17) Since upon total dehydrogenation of lumisterol$_2$ with selenium the same hydrocarbon $C_{18}H_{16}$ (γ methyl cyclo penteno phenanthrene) is obtained as from ergosterol[179] and since upon nitric acid oxidation of lumisterol$_2$ and of ergosterol the same toluene tetracarboxylic acid is obtained[180] (see page 3o6) it must be concluded that the original ring system of ergosterol is also the ring system of lumisterol$_2$ and that the system of conjugated double bonds is present in one ring which can be only ring B or ring C of the sterol ring skeleton Lumisterol$_2$ upon treat

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[173] A Windaus K Dithmar and E Fernholz *A n* 493 265 (193 )
[174] H Lettré *Ibid* 511 280 (1934)
[175] K Dimroth *B* 70 1631 (1937)
[176] K Dimroth *Ibid* 68 33 (193 )
[177] A Windaus K Dithmar and E Fernholz *An* 493 265 (193 )
[178] A Guiteras Z Nakamiya and H H Inhoffen *Ibid* 494 116 (1932)
[179] H H Inhoffen *Ibid* 494, 1?2 (1932)
[180] G Ahrens E Fernholz and W Stoll *Ibid* 500 100 (193 )

ment with perbenzoic acid yields a triol, which gives only a diacetate, and upon treatment with mercuric acetate yields dehydro lumisterol₂ [181] All these reactions are in strict analogy to the behavior of ergosterol so that there is little doubt that the double bonds are in the same position as they are in ergosterol, namely, in the 5,6 and in 7,8 positions On the other hand, lumisterol₂ does not form an addition product with digitonin and does not form a bimolecular compound upon irradiation in the presence of eosin Furthermore, upon total hydrogenation a hexahydro compound is formed which is different from that obtained from ergosterol The difference cannot be due to isomerization of the 3 hydroxyl group

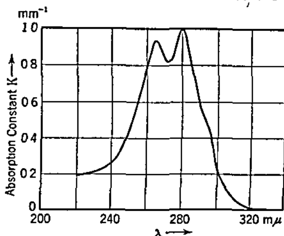



Fig 17 —Absorption spectrum of lumisterol₂ (H Brockmann)

since pyro calciferol which will be discussed later, also yields a bimolecular compound upon irradiation with eosin It must therefore be concluded that the difference between ergosterol and lumisterol is only in the steric position of one substituent on one of the asymmetrical carbon atoms 9 or 10 Actually an isomerization on carbon atom 10 is involved as will be *discussed in the section on the pyro calciferols (page 399)*

Lumisterol can be converted into vitamin $D_2$ by irradiation but is itself devoid of antirachitic efficacy

Lumisterol₃[18] and lumisterol₄[183] have been isolated from the irradiation products of 7 dehydro cholesterol and 22 dihydro ergosterol It is interesting to note that these lumisterols in contradistinction to lumisterol₂ do not form molecular addition *compounds with their corresponding* vitamins D

[181] I M Heilbron F S Spring and P A Stewart *J Chem Soc* 1935 1221 I M Heilbron and F S Spring *Chem and Ind* 54 795 (1935)

[182] A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)

[183] A Windaus and B Guntzel *Ibid* 538 1?0 (1939)

The formation of lumisterol compounds from three different provitamins D by ultraviolet irradiation allows the prediction that similar lumisterol compounds are formed by all steroids which have the system of conjugated double bonds and the steric configuration of the three provitamins ergosterol 7 dehydro cholesterol and 22 dihydro ergosterol The changes in the absorption spectrum of $\Delta^{5,7}$ androstadiene diol 3,17 upon irradiation make the existence of a lumisterol compound certain That the exact position of the double bonds in the starting material is necessary for the lumisterol formation is evident from the fact that isodehydro cholesterol upon irradiation does not form a corresponding lumisterol compound The steric specificity for the lumisterol formation is apparent since pyro calciferol and isopyro calciferol (see page 399) do not yield lumisterol derivatives

(b) **Pro-tachysterol** Pro tachysterol is obtained from lumisterol upon irradiation It has not been isolated in the pure form and is apparently not stable, but undergoes rearrangement into tachysterol as evident from spectroscopical studies [184] Thus crude irradiation products of ergosterol kept sealed in the absence of oxygen change their absorption spectrum While this dark reaction is slow at room temperature it can be brought about in a few hours by heating to 55° C The spectral changes involve the appearance of the typical absorption spectrum of tachysterol at 280 mμ

(c) **Tachysterol** [185] Tachysterol follows pro tachysterol and precedes the vitamin in the sequence of irradiation products

Tachysterol₂ can be obtained [186] from ergosterol by irradiation with the shorter wave lengths of the ultraviolet light until about 60% of the ergosterol is transformed The separation from unchanged ergosterol is effected by crystallization from methanol Tachysterol₂ is isolated from the other irradiation products by means of citraconic anhydride which forms an addition compound with tachysterol₂ Tachysterol₂ itself is obtained from the adduct by thermal decomposition While tachysterol₂ could not be obtained in crystalline form the 3 5 dinitro-4 methyl benzoic acid ester forms well shaped crystals

The constitution of tachysterol appears to be definitely established The outstanding property of tachysterol is the ease with which it is autoxidized This is considerably greater than the oxidation of ergosterol or of any of the other irradiation products Analysis of the crystallized ester established that tachysterol₂ is an isomer of ergosterol Tachysterol₂

[184] A Windaus and L Auhagen *Z phys ol Chem* 196 108 (1931)

[185] The name tachysterol has been given to this compound in recognition of its outstanding property namely the speed with which it reacts (ταχύς Gr = fast)

[186] A Windaus F v Werder and A Lüttringhaus *Ann* 499 188 (193 )

contains four double bonds that is one double bond more than ergosterol or lumisterol$_2$ This was established by the following series of reactions [187] Tachysterol acetate forms an addition product with citraconic anhydride as mentioned before The formation of this adduct requires that one double linkage disappears Catalytic hydrogenation of the adduct re

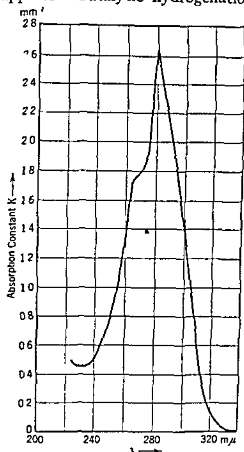



Fig 18 —Absorption spectrum of tachy sterol$_2$ (H Brockmann)

vealed that two more double bonds are present The tetrahydro tachy sterol$_2$ acetate citraconic anhydride is however not a saturated compound since the presence of one more double bond can be demonstrated upon titration with perbenzoic acid The presence of *four double linkages* requires the presence of only three rings instead of the four rings present in ergosterol and in lumisterol The fourth additional double bond which is formed by ring opening must be in conjugation to the two double bonds in ring B as *evident from the absorption spectrum* which shows maxima at 268 280 and 294 mμ (Fig 18)

[187] H Lettre *Ann* 511 280 (1934)

The ring opening during the formation of tachysterol$_2$ from lumisterol$_2$ occurs between carbon atoms 9 and 10 This has been assumed in view of the fact that in vitamin $D_2$ which follows tachysterol$_2$ in the sequence of irradiation products ring B is open between carbon atoms 9 and 10 as has been proved by oxidation experiments (see page 392) The close relationship of tachysterol$_2$ to vitamin $D_2$ has also been demonstrated by hydrogenation with sodium in alcohol both compounds yielding the identical dihydro derivative[188] (dihydro vitamin $D_2I$)

The position of the three conjugated double bonds has been established by oxidation experiments [189] Vitamin $D_2$ yields upon oxidation a ketone $C_{19}H_{32}O$ which will be discussed in the section on the Constitution of the Vitamin (see page 396) This ketone is obtained upon cleavage of the double bond between carbon atoms 7 and 8 Since the same ketone could not be obtained under similar oxidation conditions from tachysterol$_2$ it must be concluded that the latter has no double bond in the 7 8 position The positions of the three conjugated double bonds must therefore be assumed to be in the 10 5 , 6,7 and 8,9 positions

Tachysterol$_2$ has no antirachitic action and was found to be about half as toxic as vitamin $D_2$ Tachysterol$_3$ is formed from 7 dehydro cholesterol[190] and tachysterol$_4$ from 22 dihydro ergosterol[191] in a manner similar to the formation of tachysterol$_2$ from ergosterol The isolation of these compounds is carried out by means of the condensation compounds with citraconic anhydride as described None of the known tachysterols is a crystallized compound but crystalline esters have been obtained

*Dihydro tachysterol$_2$ A T 10 (Anti tetany compound No 10)* Dihydro tachysterol$_2$ is of considerable theoretical and practical importance It is prepared[192] by sodium and alcohol reduction of the 3 5 dinitro 4 methyl benzoic acid ester of tachysterol followed by saponification (Dihydro vitamin $D_2I$ is obtained in about 30% yield as a by product in the reaction ) While tachysterol$_2$ has not been obtained in the pure crystalline form the dihydro derivative$_2$ can easily be obtained in such a state

Dihydro tachysterol$_2$ is slightly active antirachitically [193] It causes an increase of the calcium concentration in the blood Tachysterol itself

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188 M Müller *Z phys l Chem* 233 2 3 (1935)

189 W Grundmann *Ibid* 252 151 (1936)

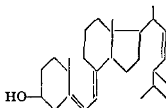
190 A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)

191 A Windaus and B Guntzel *Ibid* 538 1 0 (1939)

192 I G Farbenindustrie U S P 2 0 0 117

193 F v Werder *Z physiol Chem* 260 119 (1939)

has the same property but is only $^1/_{10}$ as active The dihydro compound is used clinically under the name 'A T 10' for the treatment of idiopathic and postoperative (hypoparathyroid) tetany [194, 195, 196] Dihydro tachysterol has the following formula, as is evident from absorption spectrum, catalytic hydrogenation and oxidative degradation reaction [197]




M p 125–127° C
Absorption maxima at 242
251 and 261 mμ

(d) **Vitamin D** The vitamins D follow the tachysterols in the sequence of photochemical reaction products from provitamins D The isolation properties and chemical constitution of the vitamins D will be described later

The vitamins D are the only photoisomers of the provitamins D with antirachitic efficacy

(e) **Toxisterol (Substance 248)** Upon further irradiation of vitamin D a toxic compound is formed which has not yet been isolated in the pure state It is characterized by its absorption spectrum which shows a maximum at 248 mμ It has no antirachitic activity[198] but is quite toxic [199, 200] There is considerable evidence that this compound is formed more readily when the irradiation is carried out in alcohol than in ether [201]

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[194] F Holtz *Merck s Jahresber* 47 0 (1934)

[195] F Holtz *Klin Wochschr* 13 104 (1934) *Deut med Wochschr* I 560 (1934) II 1830 (1934)

[196] M MacBryde *J Am Med Assoc* 111 304 (1938) J A Greene and L W Swanson *J Iowa Med Soc* 29 275 (1939) O C Pickhardt and A Bernhard *Ann Surg* 108 362 (1938) E Rose and F W Sunderman *Arch Intern Med* 64 217 (1939) L M Hurxthal and T S Claiborne *New England J Med* 220 911 (1939)

[197] F v Werder *Z physiol Chem* 260 119 (1939)

[198] A Smakula *Nachr Ges Wiss Göttingen Math physik Klasse* III 49 (19 8) C E Bills E M Honeywell and W M Cox *J Biol Chem* 80 557 (19?8) E H Reerink and A van Wijk *Biochem J* 23 I 94 (19?9)

[199] A Windaus A Lutttringhaus and P Busse *Nachr Ges Wiss Göttingen Math physik Klasse* III 150 (1932)

[200] F Laquer and O Linsert *Klin Wochschr* 12 7?3 (1933)

[201] A van Wijk and E H Reerink *Nature* 122 648 (19?8) W E Dixon and J C Hoyle *Brit Med J* II 832 (1928) L J Harris and T Moore *Biochem J* 23 261 (19 9) J C Hoyle and H Buckland *Ibid* 23 558 (1929) J C Hoyle *J Pharm* 40 351 (1930) R Kern M F Montgomery and F W Still *J Biol Chem* 93 365 (1931)

The compound is called toxisterol or substance 248 because of its outstanding ultraviolet absorption at 248 mμ (Fig 19)

(f) **Suprasterols I and II** The information available concerning the formation of the irradiation products of vitamin D is scarce It seems[202] that toxisterol and the two suprasterols I and II are formed simultaneously, but it has also been assumed[203] that toxisterol precedes the suprasterols

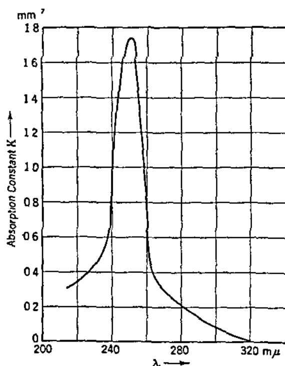



Fig 19—Absorption spectrum of toxisterol, (H Brockmann)

The two suprasterols appear to be the photochemical end products from the irradiation of the provitamins

The suprasterols, I and II have been obtained[204] from over irradiated ergosterol They have been isolated by fractional crystallization of their allophanates whereby the suprasterol, I is obtained first The suprasterols are isomers of the provitamins from which they have been obtained In the case of the suprasterols, I and II the presence of the hydroxyl group and the side chain as present in ergosterol have been proved

[202] P Setz *Z physiol Chem* 215 183 (1933)
[203] C E Bills *Physiol Rev* 15 1 (1935)
[204] A Windaus J Gaede J Köser and G Stein *Ann* 483 17 (1930)

Suprasterol$_2$ I has three double bonds according to the results obtained from catalytic hydrogenation and from titration with perbenzoic acid [205] Since neither of the suprasterols shows any absorption in the ultraviolet region above 240 mμ (Fig 20), it must be concluded that the double bonds are not conjugated The presence of only three double bonds suggests that ring closure occurred upon irradiation of vitamin D The typical

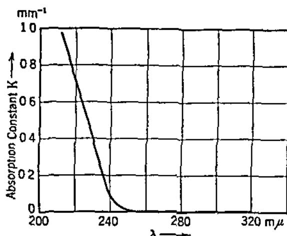
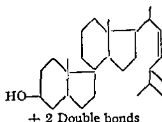



Fig 20 —Absorption spectrum of the suprasterols$_2$ (H Brockmann)

hydrocarbon, γ methyl cyclo penteno phenanthrene, which is obtained from all sterol compounds upon dehydrogenation with selenium, was not obtained from either of the suprasterols$_2$ The ring closure has therefore not resulted in the formation of the sterol skeleton

Even less is known about suprasterol$_2$ II There appear to be three double bonds, but there is no conclusive evidence to substantiate this

As long as the structure is not certain, no definite formulas can be given for the suprasterols The following spiro cyclo pentane formula has been tentatively suggested [206]




[205] M Müller Z physiol Chem 233 223 (1935)
[206] M Müller Ibid 233 223 (1935)

By over irradiation of 7 dehydro cholesterol[207] and of 22 dihydro ergosterol [208] the suprasterols$_{3 \text{ and } 4}$ respectively are formed

## VITAMINS D

The number of naturally occurring vitamins D is unknown and it is an extremely difficult task to isolate and to identify the pure vitamin D from any source Prior to the isolation of any pure antirachitic substance from natural material, various forms of vitamin D were obtained by irradiation of different provitamins Numbers were assigned to these various vitamins D in order to simplify the nomenclature Thus today we have the following series of five vitamins D with their corresponding provitamins

TABLE IV

| Vitamin D | Provitamin D | Remarks |
|---|---|---|
| Vitamin $D_1$ | Ergosterol | Vitamin $D_1$ is a molecular compound consisting of vitamin $D_2$ and lumisterol$_2$ |
| Vitamin $D_2$ | Ergosterol | Vitamin $D_2$ is also called calciferol or viosterol |
| Vitamin $D_3$ | 7 Dehydro cholesterol | Vitamin $D_3$ is also referred to as dimethyl dihydro calciferol |
| Vitamin $D_4$ | 22 Dihydro-ergosterol | |
| Vitamin $D_5$ | 7 Dehydro sitosterol | |

Actually, since at least ten different provitamins D are known ten different vitamins D also should be listed But only the four vitamins, designated vitamins $D_{2-5}$ have been prepared in essentially pure form Whether or not the four vitamins D listed above occur in nature is not known Only vitamin $D_2$ and vitamin $D_3$[209] have been isolated in the pure form from fish liver oils Furthermore another vitamin D of unknown constitution has been separated by molecular distillation from fish liver oils From the properties of this vitamin it is inferred that it is not identical with any of the above listed vitamins and it will therefore be referred to in this monograph provisionally as vitamin $D_{6(?)}$ Besides vitamin $D_{6(?)}$ five other vitamins D occur in cod liver oil as evident from

[207] A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)
[208] A Windaus and B Guntzel *Ibid* 538 1 0 (1939)
[209] H Brockmann and A Busse *Z physiol Chem* 256 ?? (1938)

molecular distillation experiments Of these six vitamins D, two are present as the major constituents two are present in lesser quantities and the last two are present only in traces [210] The distillation curves for the vitamins D from spearfish and from 'white sea bass' are different from each other and from those of cod liver oil This suggests that these fish contain different vitamins D

The complexity of the vitamins D which occur in nature can also be demonstrated by biological experiments, using different species of animals as test objects All vitamins D are primarily standardized on rats However, when tested on chicks, the naturally occurring vitamins D show quite inconsistent responses, Rat Unit for Rat Unit In order to have a standardized vitamin D with which other vitamin D preparations can be compared a special preparation of cod liver oil has been selected in the United States as a reference This material is carefully tested on rats and the response of this preparation on chicks is designated arbitrarily as a 100% activity, that is, one Rat Unit vitamin D from the U S Reference Cod Liver Oil is arbitrarily taken as one Chick Unit of vitamin D Vitamin $D_3$ also proved to be 100% chick active On the other hand vitamin $D_{6(?)}$ has only $^1/_2$ to $^1/_4$ the chicken activity of the U S Reference Cod Liver Oil Vitamin $D_2$ has practically no chicken activity The livers of some fish contain vitamins D which are much more than 100% chick active Thus vitamin D from the white sea bass" (*Cynoscion nobilis*) has been reported to be over 300% chick active and the vitamin D from the dogfish (*Squalus suckleyi*) about 230% active [211] Careful analysis of these results lead the investigators to the belief that these high chicken potencies of special fish oils are due to a real vitamin D, that is, due to at least one new, so far unknown form, and that these potencies cannot be explained on the basis of some hypothetical synergistic factor

The species specificity has also been demonstrated with turkeys A Chick Unit is not necessarily a Turkey Unit of vitamin D [212] Furthermore some evidence exists that human beings react preferentially to certain forms of naturally occurring vitamin D Thus it has been observed that seal oil is a much more effective antirachitic agent for humans than for rats [213] There is also ample evidence that vitamin $D_3$ is somewhat more effective in man than vitamin $D_2$

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[210] K C D Hickman and E L Gray *Ind Eng Chem* 30 796 (1938)

[211] C E Bills O N Massengale M Imboden and H Hall *J Nutrition* 13 435 (1937)

[212] T H Jukes and T D Sanford *Ibid* 18 71 (1939)

[213] E J Mikhlina M J Leizerovskaya and N N Milovanova *Kazan Med Zhur* 33 64 (1937) *Chem Zentr* 1938 II 3108

## 14 Occurrence

Vitamin D occurs in nature only in small amounts The living plant tissue and fresh green vegetables contain no detectable amount of this vitamin The occurrence of very small amounts of vitamin D in some species however is possible due to the presence of significant amounts of provitamin D Thus by irradiation from the sunlight a certain amount of vitamin D should be formed

From time to time it has been reported that certain plant materials contain considerable amounts of vitamin D It has not been possible however to verify these claims On the other hand it has been found that yeasts and molds grow readily on many non living plants These organisms contain significant amounts of provitamins D as discussed previously (page 345) A transition of these provitamins into vitamins D under the influence of sunlight has been proved in many cases, for example in the alleged antirachitic activity of cacao shells[214] and of hay [215]

Vitamin D occurs only in small quantities in most members of the animal kingdom [16] Only a few classes of animals and of animal products contain significant amounts

Abundant quantities of vitamin D are present in the livers and also to a certain extent in the viscera of fish The actual amount of vitamin D per gram of liver oil varies considerably with the species the season and a number of other biological factors such as age climate food supply condition of living etc Thus for example halibut livers give in the summer months a high yield in oil of low potency while in the winter months less oil with more vitamin D is obtained As a general rule it appears that fish with much body oil are the richest natural source of vitamin D The distribution of vitamin D in various fish oils may be illustrated by Table V

While the fat from fish contains relatively large amounts of vitamin D, the fat of other animals contains little or none [216] Exceptions to this general rule are certain fats from animals especially from certain birds that live upon fish Furthermore the nutritive animal material for the initial growth of those species that require vitamin D contains small but significant amounts Thus the eggs of birds are a relatively good source More specifically the yolks of hen and duck eggs contain definite amounts which are usually fairly constant (see page 418) The milk of mammals contains vitamin D Furthermore inasmuch as milk contains the opti

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[214] A W Knapp and K H Coward *Analyst* 59 474 (1934)

[215] H Steenbock I B Hart C A Elvehjem and S W F Kletzien *J Biol Chem* 66 425 (1925)

[216] P A Coppens and G A Metz *Arch neerland physiol* 18 407 (1933)

TABLE V

DISTRIBUTION OF VITAMIN D IN VARIOUS FISH OILS

| Source of oil | Zoological name | Potency I U per G |
|---|---|---|
| Bluefin tuna liver | *Thunnus thynnus* | 40 000 |
| Swordfish liver | *Xiphias gladius* | 10 000 |
| Yellowfin tuna liver | *Neothunnus macropterus* | 10 000 |
| Black sea bass liver | *Stereolepis gigas* | 5 000 |
| Bocaccio liver | *Sebastodes paucispinis* | 2 100 |
| Red rockfish liver | *Sebastodes ruberrimus* | 1 500 |
| Black rockfish liver | *Sebastodes mystinus* | 1 500 |
| China rockfish liver | *Sebastodes nebulosus* | 1 400 |
| Ling cod (not codfish) liver | *Ophiodon elongatus* | 1 300 |
| Chinook salmon liver | *Oncorhynchus tschawytscha* | 1 300 |
| Halibut liver | *Hippoglossus hippoglossus* | 1 200 |
| Rabbitfish liver | *Chilomycterus schoepfi (?)* | 1 100 |
| Striped rockfish liver | *Sebastodes elongatus* | 1 000 |
| Starry flounder liver | *Platichthys stellatus* | 1 000 |
| Boston mackerel liver | *Scomber scombrus* | 750 |
| Black cod (not codfish) liver | *Anoplopoma fimbria* | 600 |
| Pufferfish liver | *Sphoeroides maculatus* | 570 |
| Dog salmon liver | *Oncorhynchus keta* | 400 |
| Black horse mesentery | *Cycleptus elongatus* | 400 |
| Turbot liver | *Reinhardtius hippoglossoides* | 260 |
| Rex sole liver | *Errex zachirus* | 150 |
| California sand dab liver | *Orthopsetta sordida* | 120 |
| Cod liver | *Gadus morrhua* | 100 |
| Herring entire body | *Clupea harengus* | 100 |
| Yellow sole liver | *Pseudopleuronectes dignabilis* | 90 |
| Sardine entire body | *Sardinia caerulea* | 80 |
| Goosefish liver | *Lophius piscatorius* | 70 |
| Pollack liver | *Pollachius virens* | 50 |
| Menhaden entire body | *Brevoortia tyrannus* | 50 |
| Shark liver | (Sp ) | 50 |
| Salmon trimmings | *Oncorhynchus* (Sp ) | 40 |
| Turbot body minus liver | *Reinhardtius hippoglossoides* | 30 |
| Skate liver | *Raja binoculata* | 25 |
| Dogfish (Pacific) liver | *Squalus suckleyi* | 20 |
| Muddy catfish body | *Leptops olivaris* | 20 |
| Ohio perch mesentery | *Aplodinotus grunniens* | 11 |
| Buffalo mesentery | *Ictiobus cyprinella* | 10 |
| Haddock liver | *Melanogrammus aeglefinus* | 10 |
| Channel catfish mesentery | *Ictalurus punctatus* | 5 |
| Dogfish (Atlantic) liver | *Squalus acanthias* | 3 |
| Capelin entire body | *Mallotus villosus* | 3 |
| Ratfish liver | *Chimaera colliei* | 2 |
| Gray sole liver | *Glyptocephalus cynoglossus* | <1 |
| Sturgeon liver | *Acipenser rubicundus* | Nil |

From C E Bills *Physiol Rev* 15, 13 (1935)

mum amounts of calcium and phosphorus it is used extensively for supplementing human needs (see page 403) Milk products, especially dried milk and butter also contain the vitamin originally present in milk

## 15 Isolation

The isolation of vitamin D from naturally occurring fish liver oils is carried out by first isolating the unsaponifiable fraction For this purpose either the entire oil or the alcoholic extract therefrom [217] is hydrolyzed This is done under the most careful conditions for example by means of potassium hydroxide in methanol in an atmosphere of nitrogen [218] The next step consists in separating the vitamin D from the vitamin A present This can be achieved by condensing the vitamin A with maleic anhydride[219] or, better, with citraconic anhydride [220] Another separation method consists in the extraction of the mixture of vitamins A and D in hydrocarbon solution, such as benzene[221] or pentane[222] with aqueous methanol (90–95%) whereby the vitamin D remains in the hydrocarbon, whereas the vitamin A goes into the methanol solution The best method for further purification is apparently a selective adsorption on aluminum oxide according to the principle of chromatographic adsorption The position of the vitamin in the adsorption column can be detected by adding to the vitamin solution an indicator for example indicator red 33 [223] which has the same adsorption characteristics as the vitamin D The vitamin D is separated from sterols for example from cholesterol, by freezing in methanol solution[224] or by precipitation with digitonin [225, 226, 227, 228] Another method for the purification of the vitamin D is its separation from non alcoholic constituents by esterification with phthalic acid anhydride followed by fractionation of the esters [229, 230, 231] A certain purification can also be obtained by high vacuum distillation [232, 233] Final purification is achieved by esterification for example with 3 5 dinitro

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[217] T F Zucker *Proc Soc Exptl Biol Med* 19 167 (192 ) 20 136 (1922)
[218] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)
[219] O Dalmer F v Werder and T Moll *Z physiol Chem* 224 86 (1934)
[220] A Windaus O Linsert A Lüttringhaus and G Weidlich *Ann* 492 226 (1931)
[221] H Brockmann *Z physiol Chem* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)
[222] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)
[223] H Brockmann *Z physiol Chem* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)
[224] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)
[225] H Brockmann *Z physiol Chem* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A. Busse *Ibid* 249 176 (1937)
[226] E J H Simons and T F Zucker *J Am Chem Soc* 58 2655 (1936)
[227] G A D Haslewood and J C Drummond *J Soc Chem Ind* 55 598 (1936)
[228] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)
[229] E J H Simons and T F Zucker *J Am Chem Soc* 58 2655 (1936)
[230] F Ender *Z Vitaminforsch* 2 241 (1933) 3 161 (1934)
[231] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)
[232] F Ender *Z Vitaminforsch* 2 241 (1933) 3 161 (1934)
[233] O Neracher and T Reichstein *Helv Chim Acta* 19 1382 (1936)

benzoyl chloride and purification of the dinitro benzoate by *fractional* crystallization [34] [235] or by adsorption on aluminum oxide [236] Esterification may also be carried out with isocyanic acid, followed by fractional crystallizations of the allophanates obtained [37] The free vitamin D is then obtained by saponification of the esters By utilization of these methods, vitamin $D_3$ and vitamin $D_2$ have been obtained[238] from tuna liver oil and from halibut liver oil

The method of separating *naturally occurring vitamins D by molecular* distillation is also of considerable interest [239] In order to facilitate the distillation process a constant yield oil is added to the fish liver oils (For details of this method see page 61 ) Upon distillation of such a mixture free and esterified vitamins D can be separated While the former distill around 160° C the latter pass over at 230–250° C The utilization of this method has resulted in the separation of a new vitamin D, which is *characterized by the lowest boiling point of the entire vitamin D fraction* and which will be provisionally referred to in this monograph as vitamin $D_{6\,(?)}$

The methods of isolating vitamins D from the irradiation products of the provitamins are somewhat different from the methods used for the isolation of the naturally occurring vitamins D, since a different type of by products must be eliminated The best yield of vitamins D from the irradiation of provitamins D is obtained when about 40 to 60% of the provitamin has been transformed The percentage converted can be determined approximately by crystallization of the unchanged pro vitamin D from alcohols or somewhat more accurately by precipitation with digitonin Either of these operations, or a combination of both is carried out as the first step in the actual isolation procedure to obtain the pure vitamin D The isolation of the formed vitamin D is carried out in the absence of oxygen Although vitamin D is only moderately *sensitive to* molecular oxygen at room temperature, the intermediate tachysterol which is always present in the crude irradiation products, at least in small amounts adsorbs oxygen readily with the formation of peroxides which in turn have the tendency to destroy the vitamin D Tachysterol is sepa rated from the irradiation mixture by condensation with citraconic an hydride The crude reaction product is saponified at room temperature

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[34] H Brockmann and A Busse *Naturwissenschaften* 26 122 (1938)

[235] E J H Simons and T F Zucker *J Am Chem Soc* 58 2655 (1936)

[236] H Brockmann *Z physiol Chem* 241 104 (1936) *Ibid* 245 96 (1937) H Brockmann and A Busse *Ibid* 249 176 (1937)

[37] G A D Haslewood and J C Drummond *J Soc Chem Ind* 55 ?08 (193?)

[238] H Brockmann and A Busse *Z physiol Chem* 256 2.. (1938)

[239] K C D Hickman *Ind Eng Chem* 29 1107 (1937)

and extracted with petroleum ether and water whereby the tachysterol citraconic acid adduct remains in the water phase while the vitamin D goes into the petroleum ether The vitamin D is then crystallized from acetone at low temperature and recrystallized from acetone methanol

In another method used for the isolation of vitamin D after irradiation of its provitamin a characteristic sparingly soluble ester of the vitamin such as the 3 5 dinitro benzoate[240] is prepared This can be purified by fractional crystallization and the pure vitamin D is obtained by saponification of the ester

The vitamins D form addition products very easily with other compounds especially with substances that contain hydroxyl groups This behavior is of course not characteristic for the vitamins D for it is found in the case of many chemicals and especially of sterols In the case of the vitamins D this problem is, however, of practical importance Thus the vitamins D crystallize with the solvent of crystallization and especially with water The molecular compounds which the vitamins D form with other sterols are even more annoying Thus vitamin $D_2$ forms an addition product with lumisterol$_2$ (to give the so called vitamin $D_1$) and vitamin $D_3$ forms an addition compound with the irradiation product of iso dehydro cholesterol[241] which occurs as an impurity in the synthetically obtained 7 dehydro cholesterol

## 16 Properties

All the known vitamins D in the pure state, are white odorless crystals They are soluble in fats and in the usual organic solvents, for example in ether chloroform acetone alcohol and are insoluble in water They exhibit a characteristic absorption spectrum with one maximum at 265 mμ (in hexane and in ether) the molecular extinction coefficient $\epsilon = 1\ 82 \times 10^4$ $[K = 45 - 46 \times 10^3]$ (Fig 21)

**Vitamin $D_2$**

M p 115–117 C $[\alpha]_D^{20} = +103$ in abs alcohol +82 6 in acetone +33 3 in petroleum ether +91 2° in ether

3 5-Dinitro benzoate M p 148–149 $[\alpha]_D^{?} = +5?$ in benzene

*p* Nitro benzoate M p 93 $[\alpha]_D^{20} = +104$ in chloroform

Stability Crystallized vitamin $D_2$ sealed in the absence of oxygen and stored in the absence of light at 2 C is stable over a period of many months When dissolved in olive oil and kept under similar conditions more than half of the original amount is still present after five years Vitamin $D_2$ is thermolabile Although it can be

[240] F A Askew R B Bourdillon H M Bruce R K Callow J S L Philpot and T A Webster *Proc Roy Soc (London)* B109 488 (193 )

[241] H Brockmann and A Busse *Naturwissenschaften* 26 122 (1938)

sublimed at 125 C in high vacuum decomposition occurs at this temperature The two thermal decomposition products pyro calciferol and *isopyro calciferol* are usually obtained by heating to 160–190° C

Efficacy 1 g contains 40 million International Units vitamin D

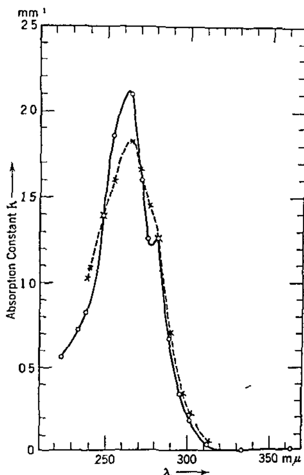



Fig 21 —Absorption spectrum of vitamin $D_2$ (O—O) and of vitamin $D_3$ (×—×) in 0 02% solution in hexane (H Brockmann)

**Vitamin $D_3$**

M p 82–83 C $[\alpha]_D^{20} = +83\ 3°$ *in acetone*

3 5-Dinitro benzoate M p 129 and 140 C (polymorphic) $[\alpha]_D^{20} = +98$ in chloroform

*p* Nitro benzoate M p 127 $[\alpha]_D^{20} = +114$ in chloroform

***Vitamin $D_4$*** [242]

M p 107–108° $[\alpha]_D^{20} = +89\ 3°$ in acetone

3 5 Dinitro benzoate M p 135–136 $[\alpha]_D^{20} = +95\ 4$ in acetone

[242] A Windaus and C Trautmann *Z physiol Chem* 247 185 (1937)

## 17 Chemical Constitution

The chemical constitution of the vitamins D is closely related to the constitution of the provitamins from which they can be derived As pointed out on page 350 the provitamins differ from each other only in the number of carbon atoms in the side chain and the degree of unsaturation The sterol skeleton is the same for all provitamins This is also true for the vitamins D derived from the provitamins D All chemically investigated vitamins D have the same constitution with the exception of different structures of the side chain

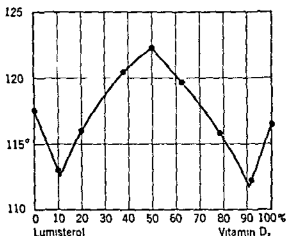



Fig 22 —Melting point diagram of lumisterol and vitamin $D_2$ (A Windaus K Dithmar and E Fernholz )

While the details of the chemical constitution of all the vitamins D have not been investigated the constitution of vitamin $D_2$ or calciferol which is derived from ergosterol has been totally elucidated The constitution of the other vitamins D can then be deduced by analogy and in the case of vitamin $D_3$ which is obtained from 7 dehydro cholesterol this deduction has been proved to be correct

### (a) *The Constitution of Vitamin $D_1$*

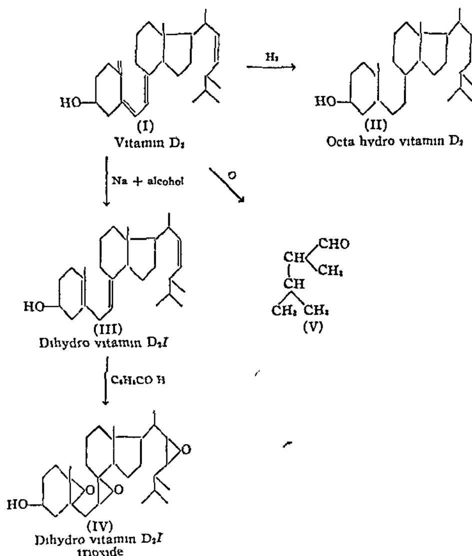
Vitamin $D_1$ has the constitution of a molecular addition product of vitamin $D_2$ with lumisterol$_2$ (see page 375) This has been shown by determination of the melting point diagram (Fig 22) obtained by mixing various amounts of the two components

### (b) *The Constitution of Vitamin $D_2$*

Vitamin $D_2$ has the empirical formula $C_{28}H_{44}O$ [243] It is therefore an isomer of ergosterol, from which it is derived The constitution of vitamin $D_2$ can consequently be linked with the structure of ergosterol (see page 350)

The oxygen present is in a hydroxyl group in the vitamin $D_2$ molecule, as is evident from the formation of esters

Vitamin D (I) contains four double bonds since upon catalytic hydro genation four mols of hydrogen are absorbed (II) [244] On the other hand



(I) Vitamin $D_2$

(II) Octa hydro vitamin $D_2$

(III) Dihydro vitamin $D_2I$

(IV) Dihydro vitamin $D_2I$ trioxide

[243] A Windaus F v Werder nd B Gschaider *Ber* 65 1106 (1932)
[244] R Kuhn and E F Möller *Angew Chem* 47 145 (1934)

titration with perbenzoic acid indicates only three of the four double linkages But the presence of the fourth double bond can be demonstrated as follows Upon reduction with sodium and alcohol two different dihydro compounds are formed—dihydro vitamin $D_2$*I* and *II* (III) Compound *I* has also been obtained by sodium reduction of tachysterol$_2$, as previously mentioned This derivative contains three double bonds [245] all of which react with perbenzoic acid to form a crystallized trioxide (IV) [246] These results indicate that vitamin $D_2$ contains one double bond more than ergosterol which in turn means that one of the four rings present in ergosterol has been opened since vitamin $D_2$ contains only three rings according to its empirical formula and the number of double bonds present

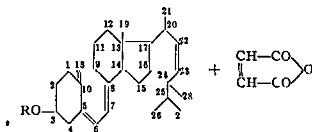
All sterols yield upon dehydrogenation with selenium the same characteristic hydrocarbon γ methyl cyclo penteno phenanthrene In accordance with the conception that vitamin $D_2$ does not contain the typical four ring system of ergosterol the typical dehydrogenation hydrocarbon could not be obtained upon reaction with selenium [247] Vitamin $D_2$ has this property in common with tachysterol$_2$ as previously described

Of the four double bonds one is in the side chain as in ergosterol, since methyl isopropyl acetaldehyde (V) is formed upon ozonolysis of the vitamin [248] The other three double bonds are in conjugation to each other as must be concluded from the typical absorption spectrum and its extinction coefficient The fact that at least two of these linkages are in conjugation is shown by the formation of an addition compound of the acetate of vitamin $D_2$ with maleic anhydride (VI) [249] Upon saponification the latter yields a dicarboxylic acid which by reaction with diazomethane is converted into a dimethyl ester (VII) This ester has been obtained in two isomeric forms Upon catalytic hydrogenation of the mixture of the two esters a dihydro compound is obtained (VIII) This compound upon oxidation with ozone does not yield the methyl isopropyl acetaldehyde which proves that the double bond in the side chain has been reduced The reaction product of the ozonolysis is however a saturated ketone of the empirical formula $C_{19}H_{34}O$ (IX) according to the analysis of its semicarbazone and its oxime The ketone contains therefore two rings and the side chain of the vitamin D, as evident from the number of hydrogen atoms present The number of carbon atoms present reveals that the two rings contain only the original side chain and the

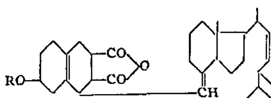
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[245] A Windaus and C Roosen Runge *Z physiol Chem* 260 181 (1939)
[246] S v Reichel and M Deppe *Ibid* 239 143 (1936)
[247] H Lettré *Ann* 511 280 (1934)
[248] A Guiteras Z Nakamiya and H H Inhoffen *Ibid* 494 116 (1932)
[249] A Windaus and W Thiele *Ibid* 521 160 (1935)

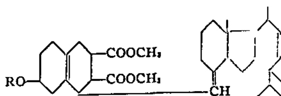
original angular methyl group between the rings C and D The keto group is therefore situated in what was originally ring C In ergosterol ring C is connected with ring B through carbon atoms 8 and 9 The



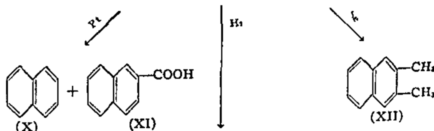
(I)
Vitamin $D_2$



(VI)

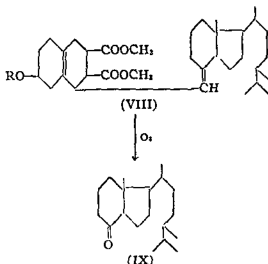


(VII)



(X) (XI) (XII)

(Formula continued on following page)



(VIII)

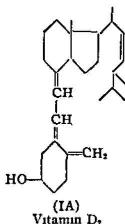
(IX)

keto group in the ozonolysis reaction product from the vitamin derivative indicates that in the vitamin molecule only one linkage exists between ring C and what was ring B in the ergosterol molecule In addition, the formation of a ketone shows that ring C is connected with the rest of the molecule by a double bond It follows that in vitamin $D_2$ ring B exists no longer but has been opened Ring cleavage has occurred between carbon atoms 9 and 10 for a keto group at carbon atom 9 in the ozonolysis reaction product is impossible since a double bond cannot exist between carbon atoms 9 and 10 due to the fact that carbon atom 10 is quaternary in ergosterol It is then concluded that one double bond in the vitamin $D_2$ molecule is in the 7,8 position The constitution of rings C and D and of the side chain is thereby established

The constitution of ring A and its connection with ring C through what was ring B in the ergosterol molecule has been elucidated as follows The dicarboxylic acid (VII) obtained by maleic anhydride addition to vitamin $D_2$ followed by hydrolysis yields upon a platinum dehydrogenation naphthalene (X) and naphthoic acid (XI) Upon dehydrogenation of the diester of the dicarboxylic acid with selenium 2,3-dimethyl naphthalene (XII) is obtained The methyl groups in the latter compound are derived from the carboxyl groups as can be shown by dehydrogenation of similar dicarboxylic acid esters [50] The results of the dehydrogenation experiments must be interpreted as meaning that by addition of the maleic anhydride to the vitamin, a hydronaphthalene derivative is formed This is possible only when the two additional unaccounted for double bonds, which

[50] W Thiele and G Trautmann *Ber* 68, 224 (1935)

are in conjugation with the double bond at the 7,8 position, are in the 5,6- and 10,18 positions The addition of the maleic anhydride occurs then on carbon atoms 18 and 6 Therefore, the constitution of vitamin $D_2$ is that indicated in formula (I) The formula may also be written in the form (IA)




(IA)
Vitamin $D_2$

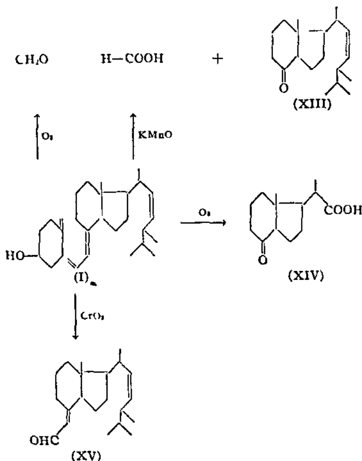
The structure of vitamin $D_2$ as deduced indicates the presence of a methylene group on carbon atom 10 instead of the methyl group present in ergosterol This was furthermore proved by oxidation experiments permanganate in acid solution yields formic acid, and ozone yields form aldehyde [251]

These oxidation experiments give further evidence on the constitution of vitamin $D_2$ Permanganate oxidation yields, besides formic acid the unsaturated ketone (XIII) which can be characterized as the semicar bazone and oxime By selective hydrogenation of the double bond there is obtained the same saturated ketone (IX) that was found in the direct oxidation of a 22 dihydro derivative as previously described Oxidation of the vitamin $D_2$ with ozone yields besides formaldehyde the keto acid (XIV), which is obtained by cleavage at the double bonds between the carbon atoms at the 7,8 and 22,23 positions By careful oxidation of vitamin $D_2$ with chromic acid rupture occurs at the double bond in the 5,6 position and the doubly unsaturated aldehyde (XV) can be isolated [52] The constitution assigned to this aldehyde is in agreement with the result of its ultraviolet spectrum, which indicates the presence of an $\alpha\beta$ un saturated oxo group

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[1] A Windaus and W Grundmann Ann 521 160 (1935)

[51] I M Heilbron R N Jones K M Samant and F S Spring J Chem Soc 1936 905

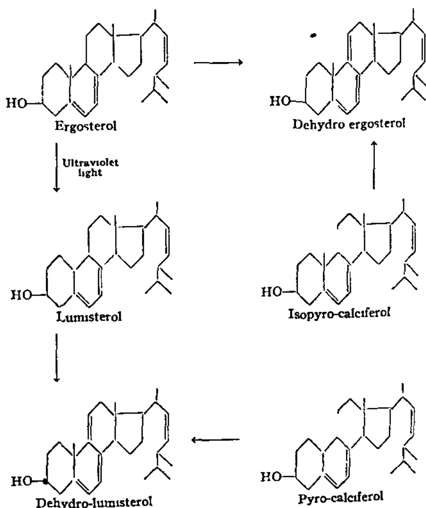



Upon heating vitamin $D_2$ (calciferol) in the absence of air at 160–190° pyro calciferol and isopyro calciferol are obtained These products are of interest in the determination of the structure of vitamin $D_2$ and also in the isomerisms involved in the photochemical activation of ergosterol and the destruction of the formed vitamin D Pyro calciferol and isopyro calciferol crystallize as molecular addition products and can be separated after acetylation by fractional crystallization Neither of the two pyro compounds shows antirachitic activity

Analysis and molecular weight determinations indicate that the two compounds are isomers of ergosterol and of vitamin $D_2$ According to the results obtained upon catalytic hydrogenation[253] and according to titrations with perbenzoic acid [254] the pyro compounds contain three double bonds Hence a ring closure is involved in the formation of the pyro compounds

[253] M Müller Z physiol Chem 233 223 (1935)
[254] I Busse Ibid 214 211 (1933)

from vitamins D Upon dehydrogenation with selenium γ methyl cyclo penteno phenanthrene is obtained,[253] showing that the basic sterol skeleton is present This indicates that the ring-closure occurred between the same carbon atoms that were involved in the ring cleavage during the activation process of ergosterol Of the three double bonds, one is inferred to be in the original 22,23 position The other two are in conjugation with each other since both pyro- and isopyro-calciferol react with maleic anhydride to yield addition compounds The double bonds are furthermore situated in the same ring, since upon nitric acid oxidation toluene 2,3,4,5-tetracarboxylic acid is obtained[255] The difference between pyro and isopyro-calciferol is evident from the fact that isopyro-calciferol forms an addition compound with digitonin whereas pyro-calciferol does not Furthermore, pyro-calciferol, but not the isopyro-




[255] H H Inhoffen *Ann* 494 116 (1932)

isomer, forms upon dehydrogenation with eosin in the presence of visible light a bimolecular compound[256] as does ergosterol Isopyro-calciferol has properties very similar to those of ergosterol in contrast to the other products in the irradiation series from ergosterol Moreover, both ergosterol and isopyro calciferol form upon dehydrogenation with mercuric acetate the identical compound, dehydro ergosterol[257] which differs from ergosterol in an additional double bond at the 9 11 position[258] On the other hand pyro calciferol when subjected to the same treatment yields a dehydro derivative which is identical with that obtained by dehydrogenation of lumisterol[259] Thus it is evident that ergosterol and isopyro calciferol differ only in the spatial arrangement at carbon atom 9 while lumisterol and pyro calciferol differ only in the spatial arrangement at carbon atom 10 This series of reactions proves furthermore that the only change involved upon conversion of ergosterol into lumisterol is an epimerization of the substituents at carbon atom 10

After the pyro calciferols were shown to be stereoisomers of ergosterol and of lumisterol it became interesting to study the changes which occur upon ultraviolet light irradiation of the pyro compounds The results[260] indicate that an entirely different reaction mechanism occurs While the pyro-compounds are not stable to irradiation no indication of the existence of intermediate products is obtained by following spectroscopically the changes occurring during the irradiation No antirachitic substance is obtained The end products of the photochemical process are obtained in crystallized form but these compounds show no absorption spectrum in the critical region between 240 and 310 mμ On heating the irradiation end products are reconverted into the two pyro-compounds This suggests that a ring cleavage similar to that obtained on irradiation of ergosterol does not occur upon irradiation of pyro-calciferol and of isopyro calciferol The changes involved appear to indicate only that a rearrangement of the two double bonds in ring B occurs so that they are no longer conjugated Upon heating the conjugation of the double bonds is restored

### (c) *The Constitution of Vitamin $D_3$*

The constitution of vitamin $D_3$ which can be derived from 7-dehydro cholesterol has been inferred by analogy with the proved constitution of vitamin $D_2$ to be as follows (I)

[256] T Kennedy and F S Spring *J Chem Soc* 1939 250
[257] A Windaus and K Dimroth *Ber* 70 376 (1937)
[258] M Müller *Z physiol Chem* 231 75 (1935)
[259] I M Heilbron F S Spring and P A Stewart *J Chem Soc* 1935 1221
[260] K Dimroth *Ber* 70 1631 (1937)

HO (I) Vitamin $D_3$ $\xrightarrow{O_3}$ (II) + (III)

Upon oxidation of vitamin $D_3$ with ozone formaldehyde,[261] [6] a satı ketone (II) and an unsaturated aldehyde (III) are obtained These been characterized by semicarbazone and by 2,4 dinitro phenyl hydı formation These oxo compounds are formed by cleavage of the 1 the 7,8 and the 5 6 double bonds exactly as ozonolysis of vitam splits these double linkages The formula of vitamin $D_3$ is th indicated

Vitamin $D_3$ upon pyrolysis yields [63] pyro vitamin $D_3$ and iso vitamin $D_3$ in strict analogy with the previously discussed formatı the pyro compounds from vitamin $D_2$ The $D_3$ compounds yield, the $D_2$ compounds upon dehydrogenation with mercuric acetate dehydro derivatives which are identical with those obtained from sterol₃ and from 7 dehydro cholesterol, correspondingly

### (d) *The Constitution of Vitamin $D_4$*

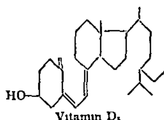
Vitamin $D_4$ is obtained by activation of 22 dihydro ergosterol view of the structure of the vitamins $D_2$ and $D_3$ the constitutic vitamin $D_4$ is believed to be the following [64]

HO Vitamin $D_4$

[261] A Windaus M Deppe and W Wunderlich *Ann* 533 118 (1937)
[262] H Brockmann and A Busse *Z physiol Chem* 256 252 (1938)
[63] A Windaus M Deppe and C Roosen Runge *Ann* 537 1 (1938)
[64] A Windaus and C Trautmann *Z physiol Chem* 247 185 (1937)

### (e) *The Constitution of Vitamin $D_5$*

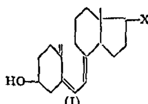
Vitamin $D_5$ is antirachitically activated 7 dehydro sitosterol and should therefore have the following constitution




Vitamin $D_5$

### (f) *The Constitution of Other Vitamins D*

On the basis of the known constitutions of the vitamins $D_2$ and $D_3$ the general formula of any vitamin D is believed to be represented by (I) wherein X stands for the side chain, the constitution of which varies with each member of the vitamin D group




(I)

It has been previously stated that not all of the members of the naturally occurring group of vitamins D have been isolated and therefore their chemical constitution is still obscure

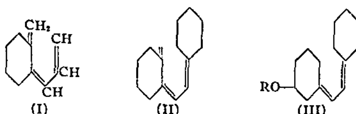
A new vitamin D has been isolated from cod liver oil (vitamin $D_{6\ (?)}$) the chemical constitution of which is not known Since it has a boiling point which is considerably lower than that of the rest of the vitamins D occurring in cod liver oil it has been suggested that it may not have a side chain at all or only a very small one [265] It is of course possible that this vitamin differs from the other known ones in stereochemical arrangement at one or more of the asymmetric carbon atoms The establishment of the structure of this new vitamin must await its isolation in crystallized form

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[265] C I Bill O N Massengale K C D Hickman and E J C y *J Biol Chem* 126 241 (1938)

## 18 Synthesis

Aside from the previously discussed partial syntheses of vitamins D by activation from provitamins no vitamin D has been obtained synthetically The total synthesis of one of the vitamins D would be of considerable theoretical interest mainly in order to study the exact stereochemical relations of the asymmetrical carbon atoms Attempts have been made to synthesize part of the molecule, that is the system of three conjugated double bonds with the attached cyclo hexane rings The following com




pounds I,[66] II[267] and III,[268] have been obtained by total synthesis The antirachitic efficacy of these compounds has not been investigated

## 19 Industrial Methods of Preparation

There are a number of different vitamin D preparations on the market Some of these originate from fish liver oils which have been processed according to the methods described for the preparation of vitamin A from fish liver oils Percomorph liver oil cod liver oil and halibut liver oil are offered for human consumption while the oils of other fish are used for animal food

Besides the sale of the natural product vitamin D prepared by activation of provitamins D is also marketed Many of the commercial products are combinations of several forms of vitamin D The activation is carried out according to the various methods described but especially by ultraviolet irradiation and by bombardment with low velocity electrons Quite a number of modifications of these two processes have been patented and are actually employed The provitamins which are used commercially have been discussed on page 365

Commercially important are the food products which are fortified with vitamin D such as bread (containing 460 International Units to the 24

[266] N A Milas and W I Alderson *J Am Chem Soc* 61 2534 (1939)
[267] K. Dimroth *Ber* 71 1333 1346 (1936) K. Dimroth and H Johnson *Ibid* 71 2658 (1938)
[268] J B Aldersley and G N Burkhardt *J Chem Soc* 1938 545

ounce loaf) breakfast cereals margarine etc Besides the actual addition of vitamin D, some foodstuffs are themselves irradiated thus gaining anti rachitic efficacy

One of the most suitable carriers for vitamin D, as far as human nutri tion is concerned is milk Milk can be fortified in its natural vitamin D content by the following methods

1 A vitamin D concentrate in oil is added to yield milk of a potency of at least 400 International Units per quart [269]

2 A solution of vitamin D in a suitable solvent, such as propylene glycol is added

3 Cows upon irradiation for example with the sun or with artificial ultraviolet light secrete a milk of increased vitamin D potency [270] that has up to 40-50 International Units per quart

4 Cows are fed with vitamin D, usually with irradiated yeast The milk secreted by such cows contains metabolized vitamin D and is standardized to contain not less than 400 International Units per quart

5 The milk itself is irradiated thereby converting the provitamin D content into vitamin D This procedure which is one of the cheapest, is also one of the most difficult since an unpleasant taste and odor are easily produced by ultraviolet irradiation [271] Quite a number of process modifications are being used and have been patented [27] The irradiated milk is standardized at 135 International Units per quart but up to 200 International Units can sometimes be produced Irradiated milk is there fore of obviously low potency

For clinical use vitamin D is marketed in oil solution or in solution with a suitable organic solvent such as propylene glycol ethers of polyhydroxy alcohols for example glycerol diethyl ether and dimethoxy trioxy hexane oxalkyl ether and alkoxyethyl ether of polyhydroxy-compounds and fatty acid amides for example ethyl acetamide Colloidal solutions of vita min D in water can be prepared that are sterilized by the application of heat

For general use by the public vitamin D is available also in the form of emulsions, tablets capsules tonics and malt preparations

## 20 Biogenesis

The biogenesis of the large amounts of *vitamin D found in fish* is a much discussed problem It seems impossible that these great quantities

[269] T F Zucker *Am J P b Health* 13 10 (1923)

[270] J E Camp on K M Henry S K. Kon and J Mackintosh *B chem J* 31 81 (1937) H R Bechtel and C A Hoppert *J Nut it on* 11 537 (1936)

[271] K G Weckel and H C Jackson *Food Resea ch* 1 419 (1936)

[27] See the re iew by W Diemair *Chem Fabrik* 14 51 (1941)

originate from provitamins in the skin of fish since not enough ultraviolet radiation is available for their conversion Furthermore as has been proved for the catfish at least,[273] fish are very sensitive to ultraviolet irradiation, and the amount of vitamin D produced under the influence of light is negligible Since, however, ultraviolet light penetrates both fresh water and sea water for about three feet,[274] the possibility has often been considered that vitamin D is produced in the plankton and in algae which in turn constitute the normal food supply of fish [275] Actually small amounts of vitamin D have occasionally been found in marine micro organisms [276, 277] It seems questionable, however, that this is a logical explanation of the presence of vitamin D in fish oil The type of vitamin D in microorganisms is at least as far as is known, not the type of vitamin D found in fish, since the vitamin D from microorganisms is relatively in active when fed to chickens, whereas the vitamin D from fish oil is generally highly active If, then, the vitamin D present in fish actually originates from the vitamin D in microorganisms, the hypothesis must be accepted that the chemical structure of the vitamin is altered in the animal organism, *which is very unlikely*

Another explanation offered is that the fish organism may contain an enzyme system which takes care of the conversion of provitamin D to vitamin D Whereas it is certain that no such enzyme system exists in humans or in chickens, the actual presence or absence of such an enzyme system in fish has not been demonstrated

Instead of the existence of an enzyme system, the so called mitogenetic radiation (Gurwitsch rays) has been postulated to cause the transition of provitamins D to vitamins D [278] It has been claimed that energy metabo lism in the growing cell causes the production of ultraviolet rays of about 190–250 mμ which play a special role in the growing tissue These rays may cause the production of vitamin D in fish and in man Since only preliminary results have been published, it seems too early to pass any judgment on this subject Some investigators deny the existence of the mitogenetic radiation [279, 280]

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[273] C E Bills *J Biol Chem* 72 751 (19 7)
[274] H. H Darby and H T Clarke *Science* 85 318 (1937)
[275] H Steenbock and A Black *J Biol Chem* 64 263 (192 )
[276] H H Darby and H T Clarke *Science* 85 318 (1937)
[277] A M Copping *Biochem Z* 28 1516 (1934) J C Drummond and E R Gunther *J Exptl Biol* 11 203 (1934)
[278] H Mai *Abhandl aus der Kinderheilkunde u ihren Grenzgeb* 1937 H 4
[279] K H Kreuchen *Angew Chem* 47 185 (1934) E N Harvey *Naturwissensch ft u* 12 16 (1924)
[280] J Levine and A H Steinhaus *Proc Am Physiol Soc* 1941 173

Finally the theory of a total vitamin D synthesis in fish has been brought forward While this theory appears plausible and possible, the experimental evidences [81] offered to prove this thesis are not conclusive

The *origin of vitamin D in higher animals* is an entirely different problem Higher animals are not able to synthesize vitamin D at least not in sufficient quantities as is evident by the incidence of rickets The previously discussed mitogenetic radiation may produce extremely small amounts of this vitamin from provitamin present in the tissue If this is the case then the susceptibility for rickets sometimes observed in infants may be explained [82, 283] on the basis of a failure to produce enough radiation or on the basis of an absence of provitamins

There is no question but that normally a considerable amount of vitamin D is gained by exposure of the animal and human body to the sun It is assumed that the vitamin D thus formed is absorbed into the blood stream However the mechanism of this reaction is not clear The skin of various organisms—of humans [284, 285] of cattle [286] of pigs [287] etc —has been shown experimentally to contain provitamin D in an amount which is at least 10 and probably 100 times greater than the amount found in the inner parts of the body Attempts have repeatedly been made to determine how deep ultraviolet radiation from the sun is able to penetrate through the epidermis but no conclusive data have been obtained While most workers believe that ultraviolet rays penetrate human skin only slightly that is 0 1 mm [288] it has been argued that the depth of penetrations depends on viability and may go through living tissue for 1 2 mm [89] It has also been stated that provitamin D may be secreted by the sebaceous glands and that the vitamin D after its formation is absorbed by the skin That the human skin is able to absorb vitamin D has repeatedly been shown[290] but it is unlikely that the sebaceous glands excrete provitamin D It has however been reported [91] that thorough washing of human skin and subsequent irradiation failed to provide enough activation to prevent serious deficiency This observation requires confirmation however

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[281] C F Bills *J Biol Chem* 72 7 1 (1927)
H Mai *Abhandl a d der Kinderheilkunde u ihren Grenzgeb* 1937 H 4o
[283] I Gl n mann *Ergeb Vitam u Hormonforsch* 1 8 (1938)
[284] A F Hess and M Weinstock *J Biol Chem* 64 181 (1925)
[285] H Hentschel and I Schneid 1 *Kl Wochschr* 9 262 (1930)
[286] A F Hess and M Weinstock *J Biol Chem* 64 181 (1925)
[287] A Windaus and F Bock *Z physiol Chem* 245 168 (1936)
[288] J Clark *Physiol Rev* 2 ? 7 (19? )
[289] W T Anderson and D I Macht *Am J Physiol* 86 3 0 (1928)
[290] M F Yoder *Z Vitaminforsch* 3 241 (1934) A C Helmer and C Jansen *Studies Inst Divi Thom* 1 83 93 (1937)
[291] A C Helmer and C Jansen *Studies Inst Divi Thomae* 1 207 (1937)

In birds the production of vitamin D by sunlight is apparently still more complicated It has been suggested[292, 293] that the preen gland excretes a provitamin D containing oil which is distributed over the feathers and after exposure to sunlight is either ingested or absorbed through the skin This could, however, not be proved experimentally[294] Furthermore, no provitamin D could be detected spectroscopically in the sterol fraction from preen glands[295] It has furthermore been shown that irradiation of the feet of chickens without preen glands cured rickets,[296] and the presence of provitamin D in the feet has been proved[295]

The site of vitamin D formation in fur bearing animals is also largely unknown It has been claimed[297] that rats which are prevented from licking their fur develop rickets On the other hand, the skin of rabbits was shown to be antirachitic, the dorsal skin more so than the ventral[298] but only in normal animals, not in those suffering from rickets

The formation of vitamin D in the organism is subject to seasonal variation due to the seasonal change of sunlight Thus the incidence of rickets in the northern hemisphere is the greatest from January to March Similar seasonal changes can also be demonstrated in the vitamin D content of milk[299] and of eggs[300] which contain maximum amounts during the summer months and minimum amounts during the late winter months

## 21 Specificity

In discussing the specificity of vitamins D a differentiation is made between compound specificity' and species specificity" Compound specificity is the response to various forms of vitamin D by one given species Species specificity is the efficacy of one given form of vitamin D for various species

The basis for all statements regarding specificity or efficacy of vitamin D is the rat test Each form of vitamin D has a certain number of rat units *per gram of the pure product These rat units are expressed* in International Units or in U S Pharmacopoeia Units which are based on the efficacy of vitamin $D_2$ This method of referring to the International

[292] H C Hou *Chinese J Physiol* 2 345 (1928) 3 171 (1929) 4 79 (1930) 5 11 (1931)
[293] W Rowan *Nature* 121 323 (1928)
[294] H R Knowles E B Hart and J G Halpin *Poultry Sci* 14 33 (1935)
[295] H R Rosenberg unpublished experiments
[296] H C Hou *Chinese J Physiol* 2 345 (1928) 3 171 (1929) 4 79 (1930) 5 11 (1931)
[297] E Reckling *Strahlentherapie* 25 568 (1927)
[298] H C Hou and E Tso *Chinese J Physiol* 4 93 (1930)
[299] J E Campion K M Henry S K Kon and J Mackintosh *Biochem J* 31 81 (1937) H L Bechtel and C A Hoppert *J Nutrition* 11 537 (1936)
[300] G M DeVaney H E Munsell and H W Titus *Poultry Sci* 12 215 (1933)

Unit of vitamin D is however only justified in a discussion regarding the compound specificity For the determination of the 'species specificity the International Units proved to be inadequate since vitamin $D_2$ has a very low efficacy on chicks There appears to exist a very definite species specificity at least for the organisms which have been investigated Thus, one rat unit is not necessarily one chick unit or turkey unit or man unit The difference between rat units and man units appears to be only slight The difference between rat units and chick units is, however, very significant In the following table the compound and species specificity are summarized for the vitamins which have been investigated

TABLE VI

COMPOUND AND SPECIES SPECIFICITY OF KNOWN VITAMINS D

| Provitamin D | Vitamin D | Rat efficacy of pure vitamin D per gram in Standard Units (1 S U = 1 million I U) | Chick efficacy in % of rat activity |
|---|---|---|---|
| Ergosterol | Vitamin $D_2$ | 40 S U | 1–3 |
| Epi ergosterol | | Unknown but active | ? |
| 7 Dehydro cholesterol | Vitamin $D_3$ | 40 S U | 100 |
| Epi 7 dehydro cholesterol | | 4 S U | ? |
| 22 Dihydro ergosterol | Vitamin $D_4$ | 20–30 S U | 20[301] |
| 7 Dehydro sitosterol | Vitamin $D_5$ | 1 3 S U | Less than 100[302] |
| ? | Vitamin $D_6$(?) | ? | 20–50 |
| 7 Dehydro stigmasterol | | 0 1 S U | ? |
| 22,23 Oxido ergosterol | | Only feebly active[303] | ? |
| Mussel provitamin D | | 40 S. U[304] | 100[304, 305] |
| $\Delta^{5,7}$ Androsta-diene diol 3 17 | | 0 1 S U[306] | |
| 3 Hydroxy $\Delta^{5,7}$ choladienic acid | | 0 1 S U[307] | |
| | Dihydro-tachysterol | 0 2 S U[308] | |

[301] F G McDonald *J Biol Chem* 114 LXV (1936)
[302] W Grab *Z physiol Chem* 243 63 (1936)
[303] A Windaus Linsert and K Buchholz quoted by K Dimroth and J Paland *Ber* 72 187 (1939)
[304] A G Boer J van Niekerk E H Reerink and A van Wijk U S P 163 659
[305] J van Niekerk and F Franken *Acta Brevia Neerland Physiol Pharmacol Microbiol* 8, 13 (1938)
[306] A Butenandt F Hausmann J Paland D von Dresler and U Meyerts *Ber* 71 1316 (1938) K Dimroth and J Paland *Ibid* 72 187 (1939)
[307] G A D Haslewood *Biochem J* 33 454 (1939) *J Chem Soc* 1938 2 4
[308] F v Werder *Z physiol Chem* 260 119 (1939)

*Compound Specificity*

The results of the compound specificity tests need further interpretation It will be recalled that none of the activation intermediates is antirachitically effective at least in the amounts investigated Thus, in order to obtain vitamin D activity ring B of the molecule must be opened between $C_9$ and $C_{10}$ and the double bonds must be in the correct positions The latter is evident from the inactivity of tachysterol$_2$ The most revealing result is the relatively great specificity of rats toward small differences in the vitamin D molecule Whereas vitamins $D_2$ and $D_3$ are equally active in rats, hydrogenation of the side chain double bond of vitamin $D_2$, which results in the formation of vitamin $D_4$ decreases the activity considerably On the other hand, activated 7 dehydro stigmasterol which differs from activated ergosterol by one additional methyl group in the side chain, is practically devoid of activity Activated 7 dehydro sitosterol, which differs from activated 7 dehydro stigmasterol only in having a saturated side chain and which differs, therefore, from activated 22 dihydro ergosterol in one additional methyl group in the side chain is somewhat more active 22,23 Oxido ergosterol shows only feeble activity, while the total absence of the side chain as in activated $\Delta^{5,7}$ androsta diene-diol 3,17[309] or the presence of a four membered carboxyl group containing side chain as in the bile acid analog of vitamins D (activated 3 hydroxy $\Delta^{5,7}$ chola dienic acid)[310] makes the compounds practically inactive It is thus evident that considerable compound specificity rests in the structure of the side chain An epimerization of the hydroxyl group in vitamin D causes a considerable decrease in efficacy, although the activity is not entirely lost ($^1/_{10}$ in the case of activated epi 7 dehydro-cholesterol) Vitamins D are furthermore only active when the hydroxyl group is free Esters and ethers of vitamins D are inactive [311] Those esters which can be hydrolyzed in the organism are active

It has repeatedly been observed that vitamin D given in milk exerts a greater antirachitic response than when the same amount of vitamin D is given in oil solution While it is now believed[312] that this effect is brought about by an optimum simultaneous intake of phosphorus and calcium as present in milk it was stated that a synergistic factor may

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309 A Butenandt E Hausmann J Paland D von Dresler and U Meinerts *Ber* 71 1316 (1938) K Dimroth and J Paland *Ibid* 72 187 (1939)

310 G A D Haslewood *Biochem J* 33 454 (1939) *J Chem Soc* 1938 224

311 A Windaus and O Rygh *Nachr Ges Wiss Göttingen Math physik Klasse* III 202 (1928)

312 B O Brien and K Morgareidge *J Nutrition* 16 91 395 (1938) *J Biol Chem* 128 LXXV (1939)

be involved A symplex formed from vitamin D and lactalbumin was held responsible for the alleged enhanced antirachitic activity [313]

A few compounds which evidently have not the basic structure of the vitamins D have also been found to exhibit antirachitic activity Thus in rats the addition of iodine or iodides to the rachitogenic diet prevents or cures rickets [314] Certain organic acids especially tartaric and citric acid [315] are able to prevent or cure rickets in rats but not in chicks [316] These effects are closely related to the acid base properties of the diet and its influence upon the development of rickets (see page 425) It has been repeatedly shown that upon treatment of cholesterol with various chemicals a compound or compounds are obtained which (without further activation) exhibit slight but definite antirachitic activity when tested on rats and which are reported to show a chicken effectiveness of over 100% The chemistry of these products is entirely unknown The activation is confined to sterols is somewhat specific to the constitution of the sterol employed[317] and does not materially activate the provitamins D The reaction is brought about by fuller s earth[318] and by sulfuric acid [319] The active compound was believed to be a monosulfonation product of cholesterol or cholesterilene However cholesterilene sulfonic acid was subsequently found to be inactive [320] Active products were obtained by the treatment of sterols with $KHSO_4$ $CuSO_4$ $ZnCl_2$ $AlCl_3$ $P_2O_5$ trichloro acetic acid, phosphoric acid etc [321]

*Species Specificity*

The *chick* is considerably more sensitive than the rat as is evident from Table VI Only a few vitamins D have been tested on chicks Vitamin $D_3$ and the vitamin derived from mussel provitamin [322] are highly active while vitamin $D_2$ is only slightly active The vitamins $D_4$ and $D_6$ (?) also have a definite but slight chicken activity As has been pointed out before

[313] C C Supplee S Ansbacher R C Bender and G E Flanigan *J Biol Chem* 114 95 (1936)

[314] R Lecoq *Compt rend* 204 1891 (1937) R Lecoq and R Duffau *Compt rend soc biol* 128 619 (1938)

[315] B Hamilton and C Schwartz *Am J Diseases Child* en 46 669 (1933) B Hamilton and M M Dewar *Ibid* 54 548 (1937) A T Shohl *J Nutrition* 14 69 (1937)

[316] J T Correll *J Nutrition* 21 515 (1941)

[317] J C Eck and B H Thomas *J Biol Chem* 128 257 (1939)

[318] C E Bills and F G McDonald *Ibid* 68 821 (1926) S K Kon F Daniels and H Steenbock *J Am Chem Soc* 50 2573 (1928)

[319] L Yoder *Science* 80 385 (1934) L Yoder B H Thomas and M Lyons *J Nutrition* 9 Suppl 6 (1935) L Yoder *J Biol Chem* 116 71 (1936) J C Eck B H Thomas and L Yoder *Ibid* 117 655 (1937) J C Eck and B H Thomas *Ibid* 119 621 (1937)

[320] A Windaus and F Kuhr *Ann* 532 52 (1937)

[321] J C Eck and B H Thomas *J Biol Chem* 119 631 (1937) 128 267 (1939)

[322] J van Niekerk and F Franken *Acta Brevia Neerland Physiol Pharmacol Microbiol* 8 13 (1938)

in the general discussion on vitamins D (page 384), the "chicken activity" is a relative figure indicating the efficiency of a given vitamin D when fed on the basis of rat units and compared with the same number of rat units of a "U S Reference Cod Liver Oil," the chicken activity of which has arbitrarily been chosen to indicate 100% activity In this connection it is also interesting to compare the chicken activity of the vitamin D in various fish liver oils with the cod liver oil (C L O) as is done in Table VII The probable experimental error (PE) involved in the determinations is also given

From Table VII it is evident that the natural fish vitamin D is a mixture of vitamins D and the conclusion has been drawn that at least one more vitamin D exists than is known today, since chicken activities as high as 200% and 300% have been observed

The *turkey* appears to have a species *specificity which differs from that* of chicks Although the difference between the chicken and turkey activity is considerably less than the difference between chicken and rat activity, it must be concluded that a chick unit of vitamin D is not necessarily identical with a turkey unit of vitamin D [323, 324] The term 'turkey unit' is used in analogy with the term "chick unit" and means the biological response of one International Unit of vitamin D from U S Reference Cod Liver Oil

The efficiency with which *man* utilizes various forms of vitamin D is much more difficult to establish There does not seem to be any question but that man can utilize vitamin D from fish liver oil, vitamin $D_2$ and vitamin $D_3$ when administered *per os* None of the other forms of vitamin D has been investigated On the basis of the available experimental data it is believed that when given by mouth vitamin $D_3$ is more active perhaps 1 5 times, than vitamin $D_2$ [325] This is especially well demonstrated with babies in the shock therapy,[326, 327, 328] and has also been repeatedly observed by feeding infants daily amounts of vitamin D prophylactically[329, 330, 331] and curatively,[332] although some investigators believe

[323] T H Jukes and T D Sanford *J Nutrition* 18 71 (1939)

[324] H M Scott J S Hughes and H W Loy *Poultry Sci* 11 177 (1932) F D Baird and D J Greene *Ibid* 14 70 (1935)

[325] P C Jeans *J Am Med Assoc* 106 2066 2150 (1936)

[326] E Graser *Z Kinderheilk* 61 716 (1940)

[327] H J Hartenstein *Monatsschr Kinderheilk* 76 Nos 3 and 4 (1937)

[328] G O Harnapp *Klin Wochschr* 17 390 (1938)

[329] A F Hess J M Lewis and H Rivkin *J Am Med Assoc* 94 1885 (1930) A F Hess and J M Lewis *Ibid* 99 647 (1932) 101 181 (1933)

[330] H Brockmann *Klin Wochschr* 16 1383 (1937)

[331] T G H Drake F F Tisdall and A Brown *J Pediatrics* 9 421 (1936)

[332] J M Lewis *J Pediatrics* 10, 155 (1937) J S Hood and I Ravitch *Ibid* 11, 321 (1937)

## Table VII

### Relative Effectiveness of Vitamin D from Different Sources for Rats and Chickens

| Oil No | Name of fish (or sterol) | Vitamins given per 100 gm diet | | | Response obtained | | | | Efficacy ratio | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A | D | | Femur ash % | I U of D/100 gm | PE % | | C L O = 100 | PE % | |
| | | I U | I U | PE % | | | | | | | |
| 1 | Halibut | 510 | 17 5 | ±7 | 45 4 | 15 1 | +9 | −9 | 86 | +11 | −11 |
| 2 | Round nosed sole | 1900 | 18 9 | 6 | 45 57 | 15 4 | 9 | 9 | 81 | 11 | 11 |
| 3 | Tuna bluefin (Cal ) | 13 | 7 2 | 7 | 35 98 | * | | | | | * |
| 3 | Tuna bluefin (Cal ) | 110 | 57 6 | 7 | 42 , | 3 3 | 8 | 8 | 16 | 11 | 1 |
| 3 x | Tuna bluefin (Cal ) | 41 | 4? 4 | 7 | 41 03 | 7 4 | 9 | 8 | 17 | 11 | 11 |
| 3 x | Tuna bluefin (Cal ) | 81 | 84 9 | 7 | 45 92 | 16 5 | 8 | 8 | 19 | 11 | 11 |
| 7 a | Tuna New England | 39 | 7 9 | 7 | 39 99 | 6 4 | 10 | 10 | 81 | 12 | 12 |
| 7 a | Tuna New England | 48 | 9 2 | 7 | 41 45 | 8 0 | 9 | 9 | 87 | 11 | 11 |
| 12 | Albacore | 9 | 20 5 | 5 | 44 42 | 12 6 | 9 | 9 | 61 | 10 | 10 |
| 12 | Albacore | 27 | 61 4 | 5 | 46 80 | 19 7 | 10 | 9 | 32 | 11 | 10 |
| 13 | Tuna yellowfin | 40 | 10 7 | 7 | 45 23 | 14 6 | 9 | 9 | 136 | 11 | 11 |
| 14 | Tuna striped | 7 | 9 6 | 5 | 35 62 | * | | | * | | * |
| 14 | Tuna striped | 43 | 56 5 | 5 | 45 12 | 14 3 | 9 | 9 | 25 | 10 | 10 |
| 15 | California bonito | 20 | 8 4 | 6 | 34 31 | | | * | | | |
| 15 | California bonito | 140 | 58 0 | 6 | 45 93 | 16 5 | 10 | 9 | 28 | 12 | 11 |
| 16 | California mackerel | 630 | 9 8 | 7 | 44 01 | 11 8 | 10 | 9 | 120 | 12 | 11 |
| 17 | Swordfish | 180 | 10 1 | 6 | 45 53 | 15 3 | 9 | 9 | 151 | 11 | 11 |
| 18 | Black sea bass | 930 | 7 8 | 7 | 41 98 | 8 5 | 9 | 8 | 109 | 11 | 11 |
| 19 | Cabrilla | 8600 | 11 0 | 7 | 40 83 | 7 2 | 9 | 9 | 65 | 11 | 11 |
| 20 | White sea bass | 2 | 2 8 | 7 | 4? 19 | 8 8 | 9 | 8 | 314 | 11 | 11 |
| 20 | White sea bass | 55 | 6 0 | 7 | 45 74 | 15 9 | 9 | 9 | 265 | 11 | 11 |
| 21 | Totuava | 470 | 10 0 | 7 | 34 90 | * | | | * | | * |
| 21 | Totuava | 2800 | 59 8 | 7 | 44 47 | 12 7 | 9 | 9 | 21 | 11 | 11 |
| 22 | Sablefish | 1600 | 6 8 | 7 | 43 59 | 11 0 | 10 | 9 | 162 | 12 | 11 |
| 22 | Sablefish | 2300 | 9 8 | 7 | 45 01 | 15 0 | 9 | 9 | 159 | 11 | 11 |
| 23 a | Lingcod | 3000 | 15 8 | 6 | 44 66 | 15 7 | 9 | 9 | 99 | 11 | 11 |
| 24 | Bocaccio | 330 | 8 7 | 6 | 44 46 | 12 7 | 9 | 9 | 146 | 11 | 11 |
| 25 | Chili pepper | 4200 | 7 2 | 7 | 40 01 | 6 4 | 10 | 10 | 89 | 12 | 12 |
| 26 | Wolffish | 680 | 9 9 | 7 | 43 08 | 10 1 | 10 | 9 | 102 | 12 | 11 |
| 27 | Basking shark | Nil | 2 | 7 | 41 94 | 8 4 | 9 | 8 | 162 | 11 | 11 |
| 28 | Dogfish | 5800 | 4 0 | 7 | 4? 50 | 9 2 | 9 | 9 | 230 | 11 | 11 |
| 29 | Pollack | 210 | 8 5 | 7 | 38 12 | 4 3 | 13 | 13 | 51 | 15 | 13 |
| 30 | Hake | 160 | 8 7 | 6 | 43 91 | 11 6 | 10 | 9 | 133 | 12 | 11 |
| 31 | Sardine | 270 | 10 7 | 7 | 43 96 | 11 7 | 10 | 9 | 109 | 12 | 11 |
| Control | Cod | 310 | 10 0 | 2 | 42 97 | 9 9 | 10 | 9 | 99 | 10 | 9 |
| Control | Cod | 2 0 | 8 0 | 2 | 42 08 | 8 6 | 9 | 8 | 108 | 9 | 8 |
| Control | Cod | ? 0 | 8 0 | | 40 89 | 7 3 | 9 | 9 | 91 | 9 | 9 |
| Control | Cod | 250 | 8 0 | 2 | 41 3 | 8 0 | 9 | 9 | 100 | 9 | 9 |
| Control | Cod | 130 | 4 0 | 2 | 38 81 | 3 1 | 11 | 11 | 128 | 11 | 11 |
| Control | Maize oil | 0 | 0 0 | 0 | 35 12 | 0 0 | | | * | | |
| Sterol | Irr ergosterol | 0 | 00 0 | | 40 00 | 6 4 | | | 3 ? | | |
| Sterol | Irr ergosterol | 0 | 403 0 | | 43 30 | 10 4 | | | 2 6 | | |
| Sterol | Irr ergosterol | 0 | 1800 0 | | 46 50 | 18 5 | | | 1 0 | | |
| Sterol | Irr cholesterol (ordinary from spinal cord) | 0 | 8 0 | 7 | 41 72 | 8 2 | 9 | 8 | 103 | 11 | 11 |
| Sterol | Irr 7-dehydro-cholesterol | 0 | 13 0 | 6 | 43 93 | 11 7 | 10 | 9 | 90 | 12 | 11 |

* Response too low for significant interpretation.

From C. E. Bills, O. N. Massengale, M. Imboden and H. Hall, *J. Nutrition* 13, 442 (1937)

that vitamin $D_2$ may be equally effective [333, 334] There are also observations to the effect that in the treatment of infantile rickets vitamin $D_2$ is not effective when administered intramuscularly, whereas vitamin $D_3$ is effective when applied in this manner [335]

## 22 Determination

### (a) *Physical Methods*

There is only one physical method which has been recommended for the determination of vitamins D, namely, the *measurement of the characteristic absorption spectrum* in the ultraviolet [336] The vitamins D have a maximum at 265 mμ This method is of course accurate only in the absence of compounds which have a similar absorption spectrum and, therefore cannot be applied for the determination of vitamins D in fish oils or in crude irradiation products of provitamins In the case of almost pure, crystalline vitamins D the spectroscopical method can be used To determine vitamins D in fish oils it has been recommended[337] to first saponify the material and to isolate the unsaponifiable part, then to separate the vitamins D from inactive sterols and from the vitamin A present by selective adsorption on aluminum oxide The vitamins D present in the residual material is then determined spectroscopically While this method may give approximate values, the accuracy is not too great due to the losses which are unavoidable during the processing

### (b) *Chemical Methods*

There is no chemical method by which vitamins D can be determined accurately or at least as accurately as by biological methods A number of color reactions for vitamins D have been proposed and have been used advantageously from time to time

1 **Aluminum-Chloride Color Reaction** [338] Vitamins D in mixture with pyrogallol in benzene solution develop a deep violet color upon heating with aluminum chloride in alcohol This method is applicable to solutions of pure vitamin D Tachysterol and suprasterol I also give this reaction

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333 M M Eliot E M Nelson D J Barnes I A Browne and R M Jenss *J Pediatrics* 9 17 (1936)

334 N Morris and M M Stevenson *Lancet* 237 876 (1939)

335 A Nitschke *Z Kinderheilk* 61 385 (1940)

336 F H Reerink and A van Wijk *Chem Weekblad* 1932 64 H Töpelmann and W Schuhknecht *Z Vitaminforsch* 4 11 (1935)

337 I Marcus en *Dansk Tids Farm* 13 141 (1939)

338 W Halden *Naturwissenschaften* 24 296 (1936) W Halden and H Tzoni *Nature* 137 909 (1936) H Tzoni *Biochem Z* 287 18 (1936)

but the color developed is somewhat weaker Vitamins D in oil solution cannot be determined by this method

2 **Antimony-Trichloride Color Reaction**[339] Vitamins D give with a saturated solution of antimony trichloride in chloroform a yellow color with a characteristic absorption maximum at 500 mμ The color developed is determined spectroscopically This method can be used for vitamin D preparations in oil and is also fairly accurate in the presence of vitamin A Tachysterol gives the same color and some other sterols give similar reactions but the color developed is weaker, and the absorption maximum is somewhat different This method has been used successfully for the determination of vitamin D preparations of natural origin and is applicable for amounts as little as 0 02–0 4 mg[340, 341, 342, 343] A modification of this method in which acetyl chloride is used in addition to the other reagents employed is said to be more accurate[344] It has also been recommended to free the solutions from sterols present by precipitation with digitonin and to absorb selectively the vitamin A present on Montana earth[345]

3 **Aniline-Color Test**[346] Liver oils and irradiated provitamins D give a red color with a mixture of aniline and hydrochloric acid

4 **Fuchsine-Sulfurous Acid Color Reaction**[347] Crystalline vitamin D acquires a violet color when added to fuchsin sulfurous acid

5 **Phosphorus Pentachloride Color Reaction**[348] Vitamin D in oil solution develops a reddish brown color with $PCl_5$ The color gradually becomes darker and is finally almost black This reaction is non specific and has even been claimed to indicate all vitamins and hormones[349]

6 **Tortelli-Jaffé Reaction**[350, 351, 352] See description under Determination of Provitamin D (page 367)

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[339] H Brockmann and Y H Chen *Z physiol Chem* 241 129 (1936)

[340] H Brockmann and Y H Chen *Ibid* 241 123 (1936)

[341] I K Wolff *Z Vitaminforsch* 7 277 (1938)

[342] A Emmerie and M van Eekelen *Acta Brevia Neerland Physiol Pharmacol Microbiol* 6 133 (1936)

K Ritsert *E Merck s Jahresberichte* 52 27 (1938)

C H Nield W C Russell and A Zimmerli *J Biol Chem* 136 73 (1940)

[343] I K Wolff *Z Vitaminforsch* 7 277 (1938)

[344] M J Shear *Proc Soc Exptl Biol Med* 23 546 (1925)

[345] A Steigmann *Kolloid Z* 45 165 (1928)

[346] W Stoeltzner *München Med Wochschr* 75 1584 (1928)

[347] F Christensen *Ibid* 75 1883 (1928)

[348] M Tortelli and E Jaffé *Ann chim appl cata* 2 80 (1914)

[349] F P Häussler and E Brauchli *Helv Chim Acta* 12 187 (1929) J M Heilbron and J S Spring *Biochem J* 24 133 (1930) V A Petrow O Rosenheim and W W Starling *J Chem Soc* 1938 677

[350] U Westphal *Ber* 72 1243 (1939)

### (c) *Biological Methods*

The biological methods for the determination of vitamins D are reliable when properly conducted, and are much superior to all physical and chemical assay methods developed so far Furthermore the amount of vitamin D needed for biological determinations is relatively small, whereas the amount needed for chemical determinations is of an entirely different order of magnitude

Rats and chicks are used as test animals for vitamin D determinations All preparations to be standardized are usually first assayed on rats The chick test takes longer time and is more costly On the other hand, as pointed out previously, the rat efficacy is no measure of the chicken efficacy of any vitamin D preparation of unknown chemical composition It is, however possible to determine once and for all the rat chicken efficacy ratio of vitamins D prepared by activation of known provitamins The ratio of such vitamins D is constant when tested under standardized conditions

The rat as such is not an ideal animal for vitamin D assays Its normal needs for this vitamin are extremely small In order to induce rickets in rats they must be fed a ration which contains calcium and phosphorus in an abnormal proportion Usually a high calcium and low phosphorus diet is used, for example, a ratio of about 5 1 It is impossible to produce rickets with a calcium phosphorus ratio of 1 1 in the diet, but a high phosphorus and low calcium diet may also be employed The absolute amounts of these minerals in the diet are of equal importance[353] (see page 421)

The chick needs considerable amounts of vitamin D even if the calcium phosphorus ratio may be normal—about 1 5 1 In conducting chick experiments it is important to avoid exposure of the birds to ultraviolet light since even small amounts of these rays cause the disappearance of the deficiency symptoms

1 **The Rat Assay Method** This method is the basis of all determinations of vitamin D and is accepted by all the leading national[353a] and international organizations concerned with vitamin D assays Young, growing rats are placed on a suitable rachitogenic diet (for example, Steenbock and Black diet 2965[354] or McCollum diet 3143[355]) and groups of rats

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[353] H B Brown A T Shohl E E Chapman C S Rose and E M Sauerwein *J Biol Chem* 98 207 (193?) A Querido *Arch neerland physiol* 20 487 (1935) A. T Sohl and S B Wolbach *J Nutrition* 11 275 (1936)

[353a] The United States Pharmacopoeia XI–1939 Supplement specifies a rat test for vitamin D assays. The assay period of this procedure is eight days the animals receiving the vitamin D only during the *first six days* The effect is evaluated according to the line test

[354] H Steenbock and A Black *J Biol Chem* 64 263 (1925)

[355] E V McCollum N Simmonds P G Shipley and E A Parks *ibid* 51 41 (1922) 47 507 (1921) *Proc Soc Exptl Biol Med* 18 275 (1921) *Am J Hyg* 1 492 (1921)

(7 to 10) are fed various amounts of the vitamin D to be tested while one group is used as reference group and is fed with U S Standard Cod Liver Oil After a period of from 6 to 10 days the animals are killed and the degree of rickets is determined by any one of the following methods

(a) *The "Line Test"* This is a curative method and for this purpose either the proximal end of the tibia[356] or the distal end of the radii or ulnae[357] is used The bones are removed from the animal and cleaned from adherent tissue A longitudinal median section is made and the section is immersed in a 2% aqueous solution of silver nitrate for one minute whereby the calcium phosphate present is converted into silver phosphate After cleaning with distilled water, the sectioned surface of the bone is exposed in water to actinic light until the calcified areas have developed a clearly defined black stain The criterion of healing is the development of a line of new calcification through the rachitic metaphysis This is determined either by visual or by photographic[358] examination of the stained section

(b) *Ash Content of the Bone* [359] This method utilizes the estimation of the ash content of the bones of the hind limbs of rats maintained on prophylactic levels of vitamin D

(c) *Radiographic Method* [360] In this test the bones of rats are examined radiographically This test is used for curative and prophylactic assays

(d) *Test for Increase of Body Weight* [361] This method is based on the increase of body weight observed on young rats on a rachitogenic diet supplemented with vitamin D (originally irradiated ergosterol) This method has not been used widely, since it is not sufficiently accurate

2 **The Chick Assay Method** The best method developed so far for the determination of vitamin D in chicks is the one recommended by the Association of Official Agricultural Chemists and is based upon the use of the percentage ash content of the tibia determined under standard conditions [362, 363] A special radiographic method has also been sug

[356] C. E Bills *J Biol Chem* 90 619 (1931)

[357] F J Dyer *Quart J Pharm Pharmacol* 4 503 (1931)

[358] P F Bech *Dansk Tids Farm* 13 253 (1939)

[359] H Steenbock and A Black *J Biol Chem* 61 405 (1924)

[360] R B Bourdillon H M Bruce C. Fischmann and T A Webster *Med Research Council Brit Special Rept Series* 1931 No 158

[361] K H Coward K M Key and G E Morgan *Biochem J* 26 1585 (1932)

[362] *J Assoc Official Agr Chem* 20 72 (1937) W B Griem *J Assoc Official Agr Chem* 21 607 (1938) Methods of Analysis A.O A.C 371 (1940)

[363] O N Massengale and C E Bills *J Nutrition* 12 429 (1936) W Grab *Z physiol Chem* 243 63 (1936)

gested[364, 365] which makes use of the differences in the tarso metatarsa distances in the joints of the legs

The relative chicken efficacy of vitamin D preparations is determined[3] by assaying the material on chicks on the basis of its rat unit content Th efficacy ratio for rats and chicks varies with the degree of calcificatio produced and is not a constant

## 23 Standards

The International Unit of Vitamin D was defined in 1934 by an International Vitamin Conference held by the League of Nations[367] On International Unit is defined as 0 025 $\gamma$ of pure crystalline vitamin D dissolved in one milligram of olive oil The properties of crystallin vitamin $D_2$ are defined

*Calciferol or vitamin $D_2$ $C_{28}H_{43}OH$*

(a) Colorless acicular crystals odorless M p 114 5–117° C (open capillary)

(b) Specific rotation

$$\text{in alcohol } [\alpha]_D^{20} = +101^\circ \text{ to } +102\ 5$$
$$[\alpha]_{5461}^{20} = +119 \text{ to } +122$$
$$\text{in chloroform } [\alpha]_D^{20} = +52^\circ$$
$$[\alpha]_{5461}^{20} = +62^\circ$$

(c) Absorption spectrum in alcohol or other suitable non absorbing solvent a *smooth curve with a maximum at 265 mμ* $E_{1cm}^{1\%}$ = *470–485*

This standard was recommended for adoption when the International Standard solution prepared according to the recommendation of the International Vitamin Conference in 1931 becomes either exhausted or unsatisfactory The 1931 Standard, although still available, should no longer be used since it gives different results on evaluation on rats by the line test method and by the bone ash method[368]

The International Unit is also the basis for the U S Pharmacopoeia Unit and in England for the M R C Unit (Medical Research Council Unit) For general use in the United States a cod liver oil preparation has been set up to serve under the term *U S P Reference Cod Liver Oil for comparative studies The vitamin D content of this U S Pharma* copoeia Reference Cod Liver Oil has been tested carefully against

[364] N Olsson *Arch Geflügelkunde* 10 11 (1936) *Kl Fyrsog afiska Sällskapets i Lund Forha dl ngar* 9 1 (1936)

[365] A Z Baker and M D Wright *Analyst* 65 3?6 (1940)

[366] O N Massengale and C E Bills *J Nutrition* 12 429 (1936)

[367] League of Nations Health Organization Memorandum on the International Standard for Vitamin D and Its Application March 1935 No 30

[368] N I Cridgeman H Lees and H Wilkinson *Analyst* 65 493 (1940)

the International Standard The latter measures properly only the activity of vitamin $D_2$ It has been pointed out before that birds are not able to utilize vitamin $D_2$ but require fish liver oil or vitamin $D_3$ The Association of Official Agricultural Chemists has therefore introduced the *A O A C Chick Unit,*' which according to its definition[369] is equal in biological activity for the chick to one unit of vitamin D in the U S Pharmacopoeia Reference Cod Liver Oil when determined according to specified conditions (see page 415)

A standard such as the U S Pharmacopoeia Reference Cod Liver Oil while it serves its purpose satisfactorily can of course only be recognized temporarily There are a number of indications that the chicken activity of cod liver oil is not the same for various batches of the oil Ultimately this oil which contains an unknown mixture of vitamins D should be replaced by a pure crystalline vitamin D, preferably vitamin $D_3$ One rat unit of this vitamin is equal to one chick unit when determined according to the method specified by the A O A C

A number of other units of vitamin D have been used or are still in use They may be summarized in the following table

| | |
|---|---|
| 1 International Unit | = 1 U S P Unit |
| | = 1 M R C Unit |
| | = 1 Coward Unit |
| | = 0 025 $\gamma$ Vitamin $D_2$ |
| | = 5–6 Poulsson Units |
| | = 1 66 Oslo Units |
| | = 6–8 Laquer Units |
| | = 2 6 Prophylactic Units |
| | = 3 25 ADMA Units |
| 1 Clinical Unit | = 12 5–17 I U |
| 1 Steenbock Unit | = 3 2 I U |
| 1 Standard Unit | = 1 000 000 I U |

## 24 Metabolism

Vitamin D can be successfully administered in various ways for example by oral ingestion by parenteral injections and by absorption through the skin[370] (See page 410 for the reported quantitative differences in the efficacy of the vitamins $D_2$ and $D_3$ when administered intramuscularly or *per os*) The environmental temperature seems to be an additional determining factor in the response of the organism to vitamin D[371]

[369] Assoc Official Agr Chem *Methods of Analysis* Fifth Ed 1940 p 371

[370] L Krenn *Münch Med Wochschr* 86 1317 (1939) E M Hume N S Lucas and H H Smith *Biochem J* 21 302 (1927)

[371] D Tomtellotte and W L Bacon *J Nutrition* 10 683 (1935)

Vitamin D is absorbed from the intestines, especially in the small gut The absorption is facilitated by the presence of fat but excess doses of fat give less favorable results [372] Vitamin D is not absorbed from mineral oil [373] Effective absorption of vitamin D is related to the presence of bile [374] Thus, animals with biliary fistula cannot utilize vitamin D when given by mouth unless administered simultaneously with a bile acid, such as taurocholic acid or desoxy-cholic acid [375] Vitamin D is not well absorbed in obstructive jaundice Vitamin D esters of acids which can be hydrolyzed in the intestines are antirachitically effective while esters which cannot be hydrolyzed also cannot be utilized [376]

From the intestines vitamin D is absorbed into the blood[377] and distributed all over the organism The healthy body contains definite amounts in the lymphs and tissue fluids Normal human blood contains from about 50 to 135 International Units per 100 cc of serum (average about 100) while rabbits were found to have a mean of approximately 50 [378] The human body and also all animals investigated, with the exception of fish, have no special storage organ for this vitamin, although substantial amounts can be found[379] in various organs such as lung, liver, spleen, brain and wherever fat is accumulated as long as there is no shortage of this vitamin in the organism The heart has consistently been found to be devoid of any stored amounts of this vitamin The liver and to a certain extent, also the viscera are special storage organs only for fish

Vitamin D is readily metabolized as is evident from the transfer of ingested vitamin D into milk of all mammals and into the eggs of birds The type of vitamin D fed is also secreted Thus, vitamin $D_2$ or $D_3$ is found in milk[380] or in eggs[381, 382] according to which form has been fed to the animal There is apparently no principal difference in the utilization of the various forms of vitamins D since vitamin D from fish liver oils [383]

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[372] A Knudson and R J Floody *J Nutrition* 20 317 (1940)

[373] M C Smith and H Spector *Proc Am Soc Biol Chem* 134 XC (1940) *J Nutrition* 20 1J (1940)

[374] W Heymann *J Biol Chem* 122 249 (1937)

[375] J D Greaves and C L A Schmidt *Ibid* 102 101 (1933) *Univ Calif Pub Physiol* 8 49 (1934)

[376] A Windaus and O Rygh *Nachr Ges Wiss Göttingen Math physik Klasse* III 202 (1928)

[377] A F Hess R F Light C N Frey and J Gross *J Biol Chem* 97 369 (1932) A F Hess M Weinstock and J Gross *Proc Soc Exptl Biol Med* 30 1357 (1933)

[378] J Warkany *Z Kinderheilk* 49 191 259 (1930) *Am J Diseases Children* 49 318 (193 ) 52 831 (1936) *Biochem Z* 293 415 (1937)

[379] H Goldblatt and K M Soames *Biochem J* 17 446 (1923) I H Page *Biochem Z* 220 420 (1930) W Heymann *J Biol Chem* 118 371 (1937)

[380] R M Bethke W E Krauss P R Record and O H M Wilder *J Nutrition* 11 21 (1936)

[381] R M Bethke P R Record C H Kirk and D C Kennard *Poultry Sci* 15 326 (1936)

[382] R M Bethke P R Record O H M Wilder and C H Kirk *Ibid* 15 336 (1936)

[383] For example R M Bethke D C Kennard and H L Sassaman *J Biol Chem* 72 695 (1927)

from activated ergosterol,[384] from activated 7 dehydro cholesterol and activated "mussel provitamin"[385] are all metabolized as described, although a small difference in the efficiency of this process for different vitamins D has been observed, that is, vitamin $D_2$ is somewhat less effectively utilized than the other forms of vitamin D[385, 386, 387] Colostrum (of cows[388]) contains from six to ten times the amount of vitamin D found in normal milk Also of special interest is the fact that vitamin D apparently can pass only in limited but definite amounts through the placental walls New born animals and babies have practically no vitamin D in their tissues[389] even though their mothers had an abundant supply On the other hand when the mother's diet was deficient, the bones and skull of the infant were found softer than normal and the teeth when erupted showed defective formation[390] All these observations suggest that a special regulatory mechanism exists which takes care of the vitamin D requirements of the embryo

Some of the vitamin D is destroyed in the organism, some is excreted[391] No quantitative balance studies are available which would indicate to what extent destruction occurs Excretion occurs only through the intestinal tract and mainly through the bile but not through the kidneys *The extent to which vitamin D is secreted through the skin is unknown*

## 25 Physiological Action

An enormous amount of work has been done in various studies to define the action and the mode of action of vitamin D The result of all these investigations has not yet developed into a well rounded picture Most work has been done either with vitamin $D_2$ or with fish liver oils and it seems quite certain that at least some differences may be found in the specific reactions of these forms of vitamin D and of vitamin $D_3$ which is considered to be the natural vitamin D of man and higher animals

[384] R F Light L T Wilson and C N Frey *J Nutrition* 8 105 (1934)

[385] J van Niekerk and Hofstra *Tijdsch Diergeneesk* 1939 66

[386] G M DeVaney H E Munsell and H W Titus *Poultry Sci* 12 15 (1933)

[387] R M Bethke P R Record O H M Wilder and C H Kick *Ibid* 15 336 (1936)

[388] J van Niekerk and M S C Bliek *Acta Brevia Neerland Physiol Pharmacol Microbiol* 9 25 (1939)

[389] A F Hess and M Weinstock *Am J Diseases Children* 27 1 (1924) *J Am Med Assoc* 83 1558 (1924) C E Bills *J Biol Chem* 72 751 (1927) C E Bills and A M Wirick *Ibid* 86 117 (1930)

[390] K W Toverud and G Toverud *Acta Paediat* (Suppl 2) 12 1 (1930)

[391] A F Hess R F Light C N Frey and J Gross *J Biol Chem* 97 369 (1932) A F Hess M Weinstock and J Gross *Proc Soc Exptl Biol Med* 30 1327 (1933)

In the broadest aspect vitamin D stimulates growth[392] While this property is apparently common to all vitamins at least as a secondary reaction, it seems of primary significance in the case of vitamin D since growth of all animals stops or is retarded in the absence of this vitamin On the basis of this property a method for the determination of vitamin D has been proposed[393] This retardation of growth is probably quite a fundamental metabolic process Thus eggs which contain an insufficient amount of vitamin D or which have been laid by hens that obtained suboptimal doses of this vitamin do not hatch[394]

The growth of bones can easily be demonstrated to be related to the action of vitamin D although the possibility should not be overlooked that the growth of cells other than bone cells may also be influenced

The physiological study of the action of vitamin D must necessarily commence with a study of the organism deprived of this vitamin and proceed to a determination of the effects brought about by the action of the vitamin and finally end with an explanation of the vitamin action

Whereas the clinical symptoms of a vitamin D deficiency will be discussed in the section on avitaminosis the broad picture of the disease and the metabolic changes must be mentioned here The clinical symptoms of the vitamin D deficiency are commonly known under the term rickets and the gross effect is obviously a disturbance of the mineral metabolism Actually the deposition of the inorganic calcium phosphorus salts in the bones is retarded or stopped entirely thus retarding or stopping the growth of bones Furthermore the inorganic material previously deposited may be withdrawn from the bones causing a considerable softening An early symptom of a vitamin D deficiency is a lowered content of phosphorus in the blood serum and later also a lowering of the calcium level is observed[395, 396] Furthermore a general decreased retention of phosphorus and at later stages of the deficiency also a decreased retention of calcium are found Thus the phosphorus metabolism is much more hampered than the calcium metabolism All these changes are not necessarily the primary ones but represent the ultimate effect of the vitamin deficiency which can be observed

To study the physiology and the mode of action of vitamin D animal experiments must be performed Experimental rickets was first obtained

[392] G Stearns P C Jeans and V Vandecar J Pediatrics 9 1 (1936) F Slyker B M Hamil M W Poole T B Cooley and I G Macy Proc Soc Exptl Biol Med 37 499 (1937)

[393] K H Coward K M Key and G E Morgan Biochem J 26 1585 (1932)

[394] J S Carver E I Robertson D Brazie R H Johnson and J I St John Washington Agr Exptl Station Bull 299 (1934)

[395] J Howland and B Kramer Am J Diseases Children 22 105 (1921)

[396] P Iversen and E Lenstrup Forhandl Forste nord Kong Paed atr (Copenhagen) 1920 83

in dogs [397] but soon rats were used[398, 399] and most of the work has been done with these animals Rats however, have usually a very low requirement for vitamin D That is, on a vitamin D deficient, but otherwise normal diet *they do not develop classical rickets* *A condition, however,* which resembles human rickets can be brought about by special rations which have a disproportion between calcium and phosphorus Thus a diet of high calcium and low phosphorus content or of low calcium and high phosphorus content produces experimental rickets in rats In addition to the ratio of calcium to phosphorus the absolute amount of each also determines the rachitogenic properties of the diet [400] Very satisfactory results were obtained with the diets Steenbock and Black No 2965 [401] and with McCollum No 3143 [402] which are similar in composition and which contain about 1 2% of calcium and about 0 25% of phosphorus the ratio of calcium to phosphorus thus being about 5 1 Rats reared on such rations develop dietary rickets that can be cured by the administration of vitamin D

Physiological studies on rats have given rise to a number of theories on the action of vitamin D None of these theories however should be generalized until confirmatory evidence from experiments with other more suitable species has been obtained In contradistinction the mode of action of vitamin D should be investigated in animals which develop rickets only by a deficiency of vitamin D and in which rickets cannot be prevented by a regulation of the calcium and phosphorus content of the diet Thus dogs [403] pigs[404] and many birds [405] especially the hen and the turkey, are useful experimental animals

The histological changes that occur during bone formation must be discussed briefly in order to understand the action of vitamin D There are three different types of tissue concerned with the growth of bones—the cartilage tissue the osteoblasts and the bone tissue During the course of normal bone growth a certain number of cartilage cells de

[397] E Mellanby *J Physiol* 52 LIII (1919) *Lancet* I 407 (1919)

[398] H C Sherman and A M Pappenheimer *Proc Soc Exptl Biol Med* 18, 193 (1920/1921) *J Exptl Med* 34 189 (1921)

[399] E V McCollum and N Simmonds *J Biol Chem* 47 111 139 175 207 235 507 (1921) See also V Korenchevsky *Brit Med J* No 3171 547 (1921) *Special Rept Sci Med Research Council* No 71 (1922)

[400] H B Brown A T Shohl E E Chapman C S Rose and E M Sauerwein *J Biol Chem* 98 207 (1932) A Querido *Arch neerland physiol* 20 487 (1935) A T Shohl and S B Wolbach *J Nutrition* 11 275 (1936)

[401] H Steenbock and A Black *J Biol Chem* 64 63 (1925)

[402] E V McCollum N Simmonds P G Shipley and E A Parks *Ibid* 51 41 (1922) 47 50 (1921) *Proc Soc Exptl Biol Med* 18 275 (1921) *Am J Hyg* 1 492 (1921)

[403] E Mellanby *J Physiol* 52 LIII (1919) *Lancet* I 407 (1919)

[404] Schoch *Mitt Lebensm Hyg* No. 1/2 176 (1939)

[405] O Wancher *Avitaminosis D hos Kyllinger* (Thesis) Copenhagen 1939

While only the relation of vitamin D to the calcium and phosphorus metabolism has been discussed, there is evidence that vitamin D is also concerned with a number of other reactions, either directly or indirectly For example, vitamin D has an *influence* upon the metabolism of other minerals, especially of magnesium and iron Vitamin D, furthermore, affects the carbohydrate metabolism, since during vitamin D avitaminosis the phosphorylation of carbohydrates is retarded [430] Vitamin D, or ultra violet light, leads to changes in carbohydrate metabolism (experiments with rats) which are very similar to those observed by administration of insulin An increase in the glycogen in the liver, and to a lesser degree in the muscle, has been observed Furthermore, the quotient carbo hydrate/lactic acid goes up in blood, liver and muscle [431] A relation of vitamin D to the fat metabolism has also been postulated

## 26 Relation to Other Vitamins and Hormones

As has been postulated in the general chapter on the interrelationship of vitamins and on the relationship of vitamins to hormones (pages 29 to 31), no single vitamin is able to exert its full action in the absence of other necessary substances The effects which occur when only vitamin D is taken out of the vitamin balance have been discussed A multiple deficiency of vitamin D *together with another or several other vitamins* has not been observed other than in the form of added symptoms from each deficiency It has been claimed that the effect of toxic amounts of vitamins D can be relieved by simultaneous administration of the vitamins of the B group such as are present in yeast [432]

At various times relations of vitamin D to different glands or hormones have been postulated The thyroid gland and the parathyroid gland have been the center of discussion It has been claimed that the administration of thyroid extracts cures rickets,[433] but it has also been claimed that thyroid intensifies the symptoms of rickets The parathyroid gland influences the calcium metabolism, but in an entirely different manner from the influence that vitamin D exerts Parathyroid raises primarily the calcium level in blood serum vitamin D, however, raises the phos

[430] E Freudenberg and A Welker *Z Kinderheilk* 41 466 (1926) H Hentschel and E Zöller *Ibid* 44 146 (1927)

[431] L Pincussen *Proc Am Soc Biol Chem* 1941 CI

[432] L J Harris and T Moore *Biochem J* 22 1461 (1928) *Lancet* I 892 (1928) R F Light G Miller and C N Frey *J Biol Chem* 84 287 (1929) E R Norris and A E Church *Ibid* 89 437 (1929) H J Jusatz *Z ges exptl Med* 87 529 (1933) *Z Vitaminforsch* 3 268 (1934) *Klin Wochschr* II 1501 (1932) Herrmann *Klin Wochschr* II 1752 (1929)

[433] A Nitschke *Z ges exptl Med* 8 236 (1932) *Z Kinderheilk* 54 233 (1933) *Klin Wochschr* 12 1793 (1933)

phorus level Parathyroid causes an increase of ionized calcium in blood, vitamin D of bound calcium Parathyroid stimulates the withdrawal of calcium from the body, vitamin D stimulates its retention All these facts caused various investigators to find either a synergistic or an antago nistic effect of parathyroid on rickets according to the type of experiments carried out It now seems that the action of both compounds is inde pendent

The secretions of various other glands for example the anterior pituitary, have been claimed to be interrelated with the action of vitamin D Thus the secretions of the lymph glands are said to influence the phos phorus and the calcium content of serum [434] It has been suggested that the ovaries influence rickets, since mothers after the parturition sometimes become rachitic [435] Finally the thymus has been reported to be involved in the functioning of vitamin D [436] All these findings and theories need further study since there is no agreement, as yet, about the relation between these effects and vitamin D

## 27 Hypovitaminosis and Avitaminosis

The clinical symptoms of vitamin D deficiency in infants and in young animals are called rickets This disease occurs most frequently in man beginning around the fourth month of age but also occurs in children of school age Early signs of the disease are noticeable continuous dis comfort and perspiration on the head Soon the typical skeletal changes especially in the ribs forearm and wrist become apparent and can be recog nized by roentgenograms The retardation in ossification of the fontanelles is especially characteristic in babies [437] The change in the bones is a lack of calcification which becomes especially noticeable at the epiphysis As a result, the ends of the long bones become greatly enlarged by excessive cartilage formation Also enlargements of the junctions between the bones especially between the ribs and the cartilages which are normally present ( rachitic rosary ) occur In more serious cases the skull is malformed The jaw may be ill shaped the teeth appear late grow too close to each other and possess ill formed enamel [438] Deficiency of vita

[434] A Nitschke *Z ges expil Med* 65 637 651 (1929) *Deut Med Wochschr* 62 629 (1936)

[435] Hanau *Korrespbl Schweiz Ärzte* 22 497 (1892)

[436] G Lucandri *Boll soc ital biol sper* 13 5 (1938) O Hirota *Folia Endoc inol Japon* 13 46 (1937)

[437] Farll E J Dalyell and Mackay *Med Research Council Rept on Rickets in Vienna Spec Rept* No 77 (19 3)

[438] M M Eliot S P Southern B A Anderson and S Arnim *Am J Diseases Children* 46 458 (1933)

min D is to a certain extent, but not altogether, responsible for the occurrence of dental caries [439] The elastic properties of the bones are generally disturbed as evidenced, for example, in rat bones by breaking load and deflection stress [440] A curvature of all bones occurs in later stages of the disease, and is especially significant in the limbs the spine, etc

A vitamin D deficiency, however, does not affect the bones exclusively but the entire body Besides the changes noted on the bones, the effects on muscles are most obvious During severe cases of rickets the muscles become weak and flabby

During vitamin D deficiency the organism is particularly susceptible to a number of infectious diseases which sometimes cause death such as bronchopneumonia, tuberculosis, infectious fevers etc It has also been reported[441] that milk fever can be prevented in cows by a vitamin D supplement fed prior to parturition

A special symptom is spasmophilia (infantile tetany) ' As previously discussed, this disease when caused by a deficiency of vitamin D is considered to indicate the beginning of healing In typical rickets, tetanic spasms often occur of either a general nature or localized in the hands and feet Sometimes cramps occur even in the heart muscles and the bronchial muscles Rickets of the adult is also called osteomalacia ' and occurs especially in women during and after pregnancy but has also been noted sporadically among men and women of all ages (osteoporosis) The general symptoms are exactly the same as those of the baby rickets namely, decalcification of the bones leading to brittleness

## (a) *Clinical Test Methods*

1 **X-Ray Determination** This method is the oldest and the one that is most commonly used but reveals only cases of avitaminosis, whereas the state of hypovitaminosis cannot be detected The roentgenographic examination of the bones of the forearm and the wrist is especially recommended both for diagnosis of rickets and for following the healing process

2 **Determination of Blood Ca and P** The normal level is 4–6 mg % of P and 9–11 mg % of calcium Any value below these is considered to indicate vitamin D deficiency The determination of Ca and of P is

[439] M Mellanby and J D King *Ergeb Vitamin Hormonforsch* 2 1 (1939) C F Taylor and C D M Day *Brit Med J* 1 919 (1939)

[440] A A Schiller H C Struck and C I Reed *Proc Am Physiol Soc* 1941 250

[441] J R Grieg *Scottish J Agr* 13 369 (1930) B Sjollema *Nutrition Abstracts & Revs* 1 621 (1932) F H Conover *Vet Med* 35 657 (1940) T M Olson *South Dakota Exptl Station Bull* 319 1938

carried out according to standardized procedures [442] The product of the values found for Ca and P is also used as criterion and should be above 30 in normal individuals This evaluation procedure is considered to give trustworthy results only in cases of severe avitaminosis and cannot be used to follow the progress during treatment

3 **Blood Phosphatase Test** [443] This test is based on the fact that the enzyme phosphatase occurs in increasing amounts in the blood during bone diseases such as rickets When rickets is healed, the amount of phosphatase in blood is again slowly but not immediately reduced

4 **Mineral Metabolism Test** [444] This test in which the phosphorus[445] and calcium[446] balance is determined is considered, in the hands of experts, to give the best data as to the state of rickets In early disease much more phosphorus is excreted than calcium At later stages calcium and phosphorus are both excreted in increased amounts The beginning of healing is characterized by a marked phosphorus retention and when healing is well under way a considerable retention of calcium and phosphorus is observed

## 28 Hypervitaminosis

Vitamins D given in large excess to any experimental animal or man are toxic It is therefore important to know the symptoms of such a D hypervitaminosis and the minimum amount of vitamin D which may cause an intoxication

The first sign of a D hypervitaminosis is digestive disorder There is a loss of appetite, vomiting and diarrhea A considerable loss of weight, an inflammation of the kidneys and finally death occur Excessive doses of vitamin D cause an increase of the calcium content of the serum which may reach a value of 17 mg % As a result metastatic calcification occurs ✓ in various organs and tissues especially in the kidneys stomach lungs heart blood vessels and bronchi At first a retention of calcium in the organism is observed but at later stages a decalcification of the skeletal

[442] For example F Müller *Z Kinderheilk* 57 243 (1935) *Z physiol Chem* 237 35 (1935) See also L Pincussen *Mikromethodik* Leipzig 1930

[443] H D Kay *J Biol Chem* 89 325 (1930) *Physiol Rev* 12 384 (1932) N Morris and O D Peden *Quart J Med* 6 211 (1937) *Arch Disease Childhood* 12 45 (1937) D J Barnes and A D Carpenter *J Pediatrics* 10 96 (1937)

[444] E Rominger *et al* *Arch Kinderheilk* 80 195 (19 7) 81 176 (19?7) E Rominger H Meyer and C Bomskov *Klin Wochschr* II 1391 (1930) II 1293 1342 (1931) *Z ges exptl Med* 73 344 (1930) 78 259 272 (1931)

[445] Phosphorus determination for example according to E Müller *Z physiol Chem* 237 35 (1935) *Z Kinderheilk* 57 243 (1935)

[446] Calcium determination for example according to B Kramer and F F Tisdall see L Pincussen *Mikromethodik* Leipzig 1930

bones sets in Finally, phosphorus is excreted and calcium *deposited in* the tissues Shortly before death an excretion of calcium through the kidneys also occurs [447]

Unfortunately, no exact data for the toxic dose can be given This is *due mainly to the fact that the toxic threshold* varies considerably among individuals As an average figure a continued daily dose of about 20,000 *International Units of* vitamin *D* per kilogram of body weight may be considered to cause intoxication in man and dogs

*Clinically, in the* so *called shock* therapy, single doses up to 1 000,000 International Units of pure crystallized vitamins D dissolved in a suitable medium have been used and no injuries have been observed *from such* treatments

The over all toxic effect of vitamin $D_2$ is believed to be somewhat greater than that of vitamin $D_3$ (studied on dogs) *After feeding dogs excessive* amounts of these two forms of vitamin D the animals were allowed to recover Functional recovery was rapid in the dog relieved *from vitamin* $D_3$ but the damage to tissues was more severe and less repaired than in the animal relieved from vitamin $D_2$ [448]

*It should, furthermore,* be noted that toxic symptoms have been observed only *when* vitamin D *was given per os or parenterally,* but never when vitamin D was supplied by ultraviolet irradiation The deleterious *effect observed on the normal organism upon over irradiation is independent* of vitaminization This suggests that a special protective mechanism *exists in the body* probably *in the skin which takes care of the* potential effects of an over irradiation of the provitamin D

## 29 Requirements

The human requirements of vitamin D[449] are difficult to estimate correctly due to an individual variation in the utilization of dietary calcium and phosphorus without added vitamin D [450] The optimum amount of vitamin D for babies children and adolescents is believed to be about 400 to 800 International Units per day provided a sufficient amount of calcium and phosphorus is offered The best combination of the necessary minerals with vitamin D is found in milk, which may be fortified with vitamin D The need of adults for vitamin D appears to be somewhat smaller but exact data are not available Pregnant and lactating women[451]

[447] C A Ashford *Biochem J* 24 661 (1930) L J Harris and J R M Innes *Ibid* 25 367 (1931)
[448] A F Morgan J B Hendricks and R M Freytag *Proc Am Soc Biol Chem* 1941 XCII
[449] P C Jeans and G Stearns *J Am Med Assoc* 111, 703 (1938)
[450] H A Hunscher F C Hummel and I G Macy *Proc Soc Exptl Biol Med* 35 189 (1936)
[451] K W Toverud and G Toverud *Acta Paediat* (Suppl 2) 12 1 (1936)

are advised to take at least 800 I U per day Babies born prematurely and twins need increased amounts (See also page 613 for the recommended daily allowances as established by the National Research Council )

These requirements as stated pertain only to the ingested forms of vitamin D and to the optimum daily intake of normal organisms for protection against rickets To cure rickets in infants a daily dose of from 500 to 1500 International Units is usually given The method of supplying the body only once every three to six months instead of every day is applied in the so called shock therapy Massive doses of 200 000 to 1 000 000 International Units have been recommended and used for this purpose[45, 453, 454]

The vitamin D requirement of poultry has been studied extensively because of its practical importance and is usually expressed in vitamin D content per pound of feed It has been recommended to incorporate about 180 A O A C chick units of vitamin D per pound of total feed for growing chicks, while for the laying stock 360 A O A C chick units and for the breeding stock 540 A O A C units are required

The vitamin D requirement of species other than man and poultry has not been investigated quantitatively Some data have been presented which indicate that dogs especially dogs of large breeds such as Great Dane, Setter Airdale and German Shepherd need considerably more vitamin D than small breeds such as Terrier and Spaniel[455] In terms of vitamin $D_2$ small breeds need about 28 U S Pharmacopoeia Units per kg of body weight while large breeds need ten times this amount or even more It seems conceivable that this species difference is due to the compound specificity of the vitamin $D_2$ used and that the vitamin D requirement of dogs is more uniform and considerably lower when other forms of vitamin D such as vitamin $D_3$ are fed In dogs a greater tendency toward rickets has been observed in males than in females[456]

Swine and especially young pigs need vitamin D and when reared on a low vitamin D intake became definitely rachitic especially in the winter months[457] Vitamin D given to pigs resulted also in a considerable decrease in the amount of feed necessary for maximum growth[458] A daily ad

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[453] S Gunnarson *Acta Paedi t* 25 69 (1939)

[454] J Ström *Ibid* 25 251 (1939)

[454] G O Harnapp *Kli Wochschr* 15 1043 (1936) *Monatsschr K d rhe lk* 71 193 (1937) *Klin Wochschr* 17 390 (1938)

[455] A F Morgan *No th Am Vet* 21 462 (1940)

[456] C R Stockard *Am J Dis ases Ch ldren* 36 310 (19 8)

[457] G Bohstedt R M Bethk B H Edgi gton and W L Rob nson *Oh o Exptl Statio Bull* 395 (1026) W C Skelley *V J Exptl Stat on B ll* 661 (1939)

[458] R D Sinclair *Sc Ag* 9 629 (1979)

ministration of approximately 110 U S Pharmacopoeia Units vitamin $D_2$ has been suggested for pigs per kg of body weight Sheep also benefit from an intake of vitamin D[459] and many reports have been published showing the need of cattle and especially of calves for vitamin D[460] A daily intake of about 200 U S Pharmacopoeia Units vitamin D per kg of body weight has been suggested for these animals For horses 200 to 2000 U S Pharmacopoeia Units have been given per kg of body weight for the cure of rickets[461]

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459 D W Auchinachie and A H H Fraser *J Agr Sci* 22 560 (1932)

460 I W Rupel G Bohstedt and E B Hart *Wisconsin Expt Station Research Bull* 115 (1933) N W Hilston (Thesis) The Pennsylvania State College 1937 T W Gulliken L S Palmer and W L Boyd *Minnesota Agr Stat Techn Bull* 105 (1935)

461 J H Kinter and R L Holt *Philippine J Sci* 49 1 (1932)

# THE GROUP OF VITAMINS E

# THE GROUP OF VITAMINS E

The physiological effect of vitamin E is brought about by a series of naturally occurring compounds, which are chemically very closely related being homologs and isomers of each other Three different compounds have been isolated which are designated as α , β and γ tocopherol The possibility that other vitamin E factors may occur in the animal or plant organism cannot be excluded but no definite proof for the existence of other compounds can be offered

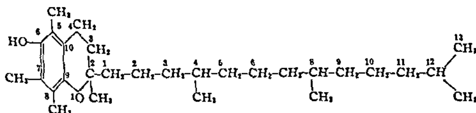
## 1 Nomenclature and Survey

**Names**

Vitamin E[1, 2]
Tocopherols[3] (tokos (Greek) meaning childbirth phero (Greek) meaning to bear)
Anti encephalomalacia vitamin
Factor X[1]
Antisterility factor
Reproductive vitamin
Sterilamine[4]
Fertility vitamin.

**The members of the group of vitamins E**

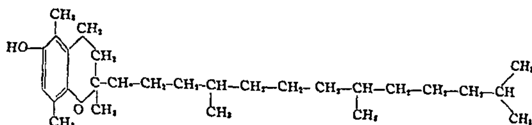
1 *α Tocopherol* $C_{29}H_{50}O_2$
Synonym 5 7 8 Trimethyl tocol[5]




[1] H M Evans and K S B shop Sc ence 56 650 (192 ) *Am J Physiol* 63 396 (1922) *J Am Med Assoc* 81 889 (1922) *J Metabolic Research* 1 319 335 (1922)
[2] B S re *J Biol Chem* 59 19 (19 4)
[3] H M Ev ns O H Emerson and G A Emerson *Ibid* 113 319 (1936)
R L Jones *Science* 68 480 (19 8)
P Karrer and H Fritzsche *Helv Chim Acta* 21 1234 (1938) proposed the t rm tocol for the general class of tocopherols w thout ubstituents in the benzene n cleus. The ngle members are nd cated by follow ng the Geneva nomenclature principle for chroman Accord ngly α tocopherol is 5 7 8 t imethyl tocol β tocopherol is 8 d methyl tocol etc

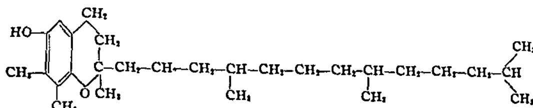
2 *β Tocopherol* $C_{28}H_{48}O_2$

Synonyms Cumo tocopherol[6] neo tocopherol *p* xylo tocopherol[7] 5 8 dimethyl tocol[6]



3 *γ Tocopherol* $C_{28}H_{48}O_2$

Synonyms *o* Xylo tocopherol[7] 7 8-dimethyl tocol[6]



**Efficacy**

1 g α tocopherol = 400 Rat Units
1 g β tocopherol = 200 Rat Units
1 g γ tocopherol = 200 Rat Units
1 g *synthetic racemic tocopherol acetate (dl α tocopherol acetate)* = 1000 International units

## 2 Chronology

1920 MATILL and CONKLIN[8] observed disturbances in the reproduction of rats on special milk diets

1922 EVANS and BISHOP[9] reported failure of rats to reproduce when on a purified diet and recognized the missing factor as a vitamin

1927 EVANS and BURR[10] recognized that vitamin E is non saponifiable

1928-1930 EVANS and BURR[11] and GOETTSCH and PAPPENHEIMER[12] described the

[6] W John *Z physiol Chem* 250 11 (1937)

[7] According to a suggestion by O H Emerson and L I Smith *J Am Chem Soc* 62 1869 (1940) the substitution in the aromatic ring of the tocopherol structure is indicated in the name of the tocopherol by a prefix which describes the substitution in terms of simple benzene derivatives such as o m and *p* xylo-tocopherol tolu tocopherols etc

[8] H A Matill and R E Conklin *J Biol Chem* 44 137 (1920)

[9] H M Evans and K S Bishop *Science* 56 650 (1922) *Am J Physiol* 63 396 (1922) *J Am Med Assoc* 81 889 (1922) *J Metabolic Research* 1 319 335 (1922)

[10] H M Evans and G O Burr *Mem Univ Calif* No 8 (1927)

[11] H M Evans and G O Burr *J Biol Chem* 76 273 (1928)

[12] M Goettsch *Proc Soc Exptl Biol Med* 27 564 (1930) A M Pappenheimer *Ibid* 27 567 568 (1930) M Goettsch and A M Pappenheimer *J Exptl Med* 54 145 (1931)

occurrence of a specific muscular dystrophy in rats rabbits and guinea pigs on a vitamin E-deficient diet

1936 EVANS EMERSON and EMERSON[13] isolated two different vitamins E (α and β tocopherol) in the form of crystallized esters

1937–1938 FERNHOLZ[14] [15] elucidated the chemical structure of α tocopherol

1938 KARRER followed by L I SMITH and by TODD synthesized tocopherol[16]

## 3 Occurrence

The group of vitamins E occurs predominantly in plant materials The animal organism contains only small amounts

The best natural source of vitamins E is vegetable oils such as wheat germ oil which contains especially high amounts Varying amounts of this group of vitamins are found in cottonseed oil[17] rice germ oil and other seed germ oils[18] Olive oil does not contain any vitamins E arachis oil contains traces[19] Lettuce alfalfa etc contain considerable amounts oranges and bananas small amounts Animal materials contain little vitamin E The highest amount has been found[20] in livers (of horse and cattle but not of rats[21] ) and small amounts are present in the muscles heart kidneys placenta milk and eggs Fish liver oils which are especially rich in the vitamins A and D are poor in vitamin E

Different vitamins E or different mixtures of vitamins E occur in the various natural sources Thus α and β tocopherols have been found in wheat germ oils but not always in the same relative proportions In European oil the β compound is the main principle while in American sources the α form is the predominant factor with smaller quantities of γ tocopherol[23] Cottonseed oil palm oil and corn oil[24] contain predominantly γ tocopherol besides small amounts of α tocopherol

The tocopherols occur in the free form[25] at least to a considerable extent in the seed oils It is believed that some occur esterified but no definite data concerning this are available

[13] H M Evans O H Emerson and G A Emerson *J Biol Chem* 113 319 (1936)
[14] F Fernholz *J Am Chem Soc* 59 1154 (1937)
[15] F Fernholz *Ibid* 60 700 (1938)
[16] See the literature references on page 44
[17] H S Olcott *J Biol Chem* 107 471 (1934)
[18] H M Evans and G O Burr *Proc Natl Acad Sci U S* 11 334 (1925) H S Olcott and H A Mattill *J Biol Chem* 104 423 (1934)
[19] A L Bacharach E Allchorne and H E Glynn *Biochem J* 31 2287 (1937)
[20] P Karrer W Jaeger and H Keller *Helv Chim Acta* 23 464 (1940)
[21] W F J Cuthbertson R R Ridgeway and J C Drummond *Biochem J* 34 34 (1940)
[22] T Moore A J P Martin and K R Rajagopal *Soc Chem Ind Food Group* 1939 41
[23] A R Todd F Bergel and T S Work *Biochem J* 31 2257 (1937)
[24] O H Emerson G A Emerson and H M Evans *Science* 89 193 (1939)
[25] A R Moss and J C Drummond *Biochem J* 32 1953 (1938)

The tocopherols occur naturally together with other compounds of un known constitution which are even stronger antioxidants than the vitamins E and which protect the vitamins against oxidation

## 4 Isolation

The isolation of the vitamins E is usually carried out by first isolating the unsaponifiable part of the vitamin E containing material Wheat germs for example are dried and extracted with an organic solvent, such as chloroform, ether, etc Another method is to press the germs, to collect the oil and to extract the residues The total fats are then saponified at room temperature with, for example, 20% alcoholic potassium hydroxide in *the absence of oxygen and the non saponifiable* part (approximately 5% of the oil) is extracted with an organic solvent Sterols constitute up to 90% of the non saponifiable material and are separated by crystallization from suitable solvents such as alcohols, pentane, etc The last traces are then removed by precipitation with digitonin The remaining oil can be purified by distillation, the vitamin E containing fraction being carried over at 200–250° C at 0 1 mm pressure [26, 27, 28] Some of the vitamin is, however lost in this procedure Some purification of the unsaponifiable mass can be achieved by partition between different solvents, such as methanol and petroleum ether,[29, 30] whereby the vitamin goes into the latter By utilization of the principle of the chromatographic adsorption on aluminum oxide, a certain further purification can be achieved [31, 32]

Instead of first isolating the non saponifiable part the vitamins E can also be obtained in improved yields directly from, for example, wheat germ oils, by application of the principle of the chromatographic adsorption method [33]

Final isolation of the vitamins E is achieved by precipitation in the form of a crystallized ester The allophanates are especially useful for this pur pose [34] By fractional crystallization of these esters, the members of the group of vitamins E are separated *The most insoluble fraction represents* the α tocopherol and from its mother liquors the allophanates of the β

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[26] H S Olcott *J Biol Chem* 107 471 (1934)
[27] H S Olcott and H A Matill *Ibid* 104 4?3 (1934) H S Olcott *Ibid* 110 695 (1935)
[28] F W Quackenbush H L Gottlieb and H Steenbock *Ind Eng Chem* 33 1276 (1941)
[29] H M Evans O H Emerson and G A Emerson *Ibid* 113 319 (1936)
[30] A R Todd F Bergel and T S Work *Biochem J* 31 2?57 (1937)
[31] J C Drummond E Singer and R J MacWalter *Ibid* 29 456 2510 (1935)
[32] J C Drummond and A A Hoover *Ibid* 31 1852 (1937)
[33] A R Moss and J C Drummond *Ibid* 32 1953 (1938)
[34] H M Evans O H Emerson and G A Emerson *J Biol Chem* 113 319 (1936)

isomer are recovered The γ isomer has been isolated from a different source by the same technic [35]

An efficient separation of the α and β tocopherols is also achieved by the adsorption method

## 5 Properties

The α, β and γ isomers of tocopherol are oils which have not been obtained in the crystalline state Certain esters, however such as the

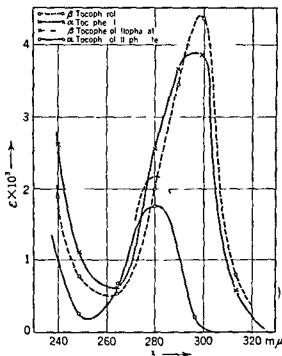



Fig 23 —Absorption spectra of α and β tocopherol and of their allophanates (H Rudy in W Stepp *Ernahrungslehre*)

allophanates p nitrophenyl urethanes and 3 5 dinitro benzoates are crystalline The tocopherols have a characteristic absorption spectrum in the ultraviolet with a maximum at 295 mμ which is displaced to 285 mμ in the esters, such as the acetates The extinction coefficient for α tocopherol for example, is $E^{1\%}_{1\,cm}$ = approximately 77 while the coefficient of the esters is reduced to approximately 42 (Fig 23)

In the absence of oxygen the vitamins E are stable to heat treatment up to 200° C and are not affected by sulfuric or hydrochloric acid up to 100° C

[35] O H Emerson G A Emerson and H M Evans *Science* 83 4 1 (1936)

Alkali destroys the vitamins of this group only very slowly, so that they can be obtained by alkaline saponification The tocopherols are, however, quite sensitive to oxidation, which process destroys the biological activity

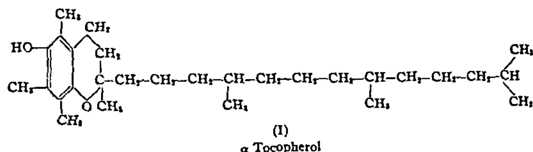
The vitamins E are soluble in all lipoid solvents, but are insoluble in water They are fairly stable to visible light, but are destroyed readily by ultraviolet light [36]

The tocopherols are effective antioxidants, the $\gamma$ isomer being more effective than the $\beta$ isomer, which in turn is more effective than the $\alpha$ isomer [37] Thus the antioxidant power is the reverse of the vitamin activity and is dependent upon the presence of a free phenolic hydroxyl group, which is not necessarily important for the vitamin action (see page 449)

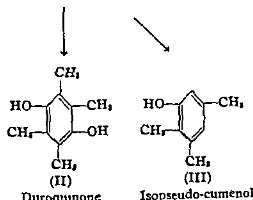
## 6 Chemical Constitution

### (a) α-Tocopherol

$\alpha$ Tocopherol has the empirical formula $C_{29}H_{50}O_2$ One of the oxygens is present in the form of a free hydroxyl group since the vitamin readily forms esters[38, 39, 40] and ethers The phenolic character of this hydroxvl group



(I)
α Tocopherol



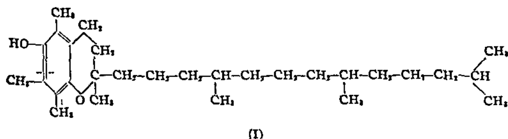
(II) Duroquinone

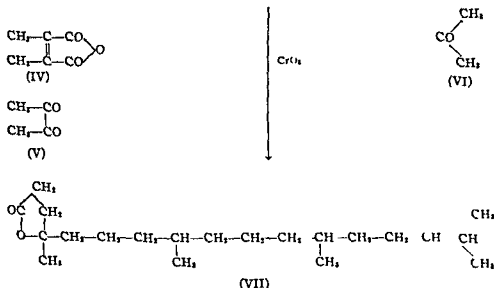
(III) Isopseudo-cumenol

[36] J C Drummond E Singer and R J MacWalter *Biochem J* 29 456 2510 (1935)
[37] H S Olcott and O H Emerson *J Am Chem Soc* 59 1008 (1937)
[38] H. S Olcott *J Biol Chem* 107 471 (1934)
[39] H S Olcott *Ibid* 110 695 (1935)
[40] H S Olcott and H A Mattill *Ibid* 104 423 (1934)

was suspected on the basis of the change of the absorption spectrum upon esterification [41] Upon pyrolysis α tocopherol yields[42] duroquinone (II), while isopseudo cumenol (pseudo cumenol 6) (III) is obtained by heating with hydriodic acid [44] Upon hydrogenation four mols of hydrogen are absorbed [43]

Further insight into the structure of α tocopherol was obtained by oxidative degradation with chromic acid whereby the following reaction products were isolated dimethyl maleic anhydride (IV) diacetyl (V), acetone (VI), a $C_{21}$ lactone (VII), a $C_{18}$ ketone (VIII) and a $C_{16}$ acid (IX) [45]



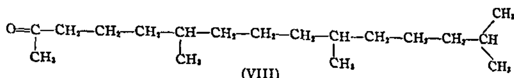



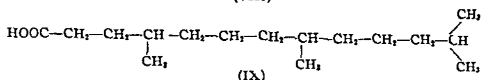

[41] W John *Z physiol Chem* 250 ii (1937) W John E Dietzel and P Günther *Ibid* 252 208 (1938) *Naturwissenschaften* 26 366 (1938) W John *Z physiol Chem* 252 2 2 (1938)

[42] E Fernholz *J Am Chem Soc* 59 1154 (1937)

[43] F Bergel A R Todd and T S Work *J Chem Soc* 1938 253

[44] W John *Z physiol Chem* 250 ii (1937) W John F Dietzel and P Günther *Ibid* 252 208 (1938) *Naturwissenschaften* 26 366 (1938) W John *Z physiol Chem* 252 222 (1938)




Of these, the $C_{21}$ lactone is of greatest importance The hydroxy acid from which the lactone is derived, lactonizes readily thus indicating that the hydroxyl group is in either $\gamma$- or $\delta$ position to the carboxyl group The methyl ester of the hydroxy acid could not be oxidized to a keto acid which proves that the hydroxyl group is tertiary Additional evidence for this fact is furnished by the difficulty encountered upon attempted esterification of this hydroxyl group The $C_{16}$ acid (IX) served for a determination of the side chain methyl groups, which indicated the presence of three In analogy to many naturally occurring compounds of the terpene family which follow the isoprene rule, formula (IX) was postulated for the $C_{16}$ acid and formula (I) for $\alpha$ tocopherol The correctness of this formula at least as far as the structure of the aliphatic side chain is concerned, is evident from the successful synthesis of this vitamin from phytol

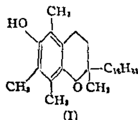
The formula of $\alpha$ tocopherol (I) shows the presence of a chroman ring system That such a ring system and not a coumaran structure occurs was proved[44] by careful oxidation with ferric chloride or with silver nitrate which yielded a yellow quinone (X)[45] This undergoes reduction to a *hydroquinone, which is a hydroxy quinol ('Tocopheryl quinol') (XI)* Upon esterification of the phenolic hydroxyl groups the remaining aliphatic hydroxyl group was investigated and showed all the properties of a tertiary hydroxyl group in oxidation and esterification experiments A coumaran compound (XII) on the other hand, should have given a secondary hydroxyl group (XIII) The hydroxy quinol (XI) can be reconverted into $\alpha$ tocopherol by treatment with strong mineral acids

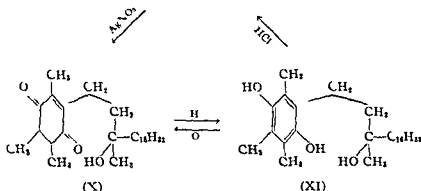
The tocopherols have three asymmetric centers namely at carbon atoms 2 4′ and 8′ It is still an open question whether or not each of the naturally occurring tocopherols represents one of the eight possible isomers Available evidence indicates that the natural products may be racemic about all three asymmetric centers[46]

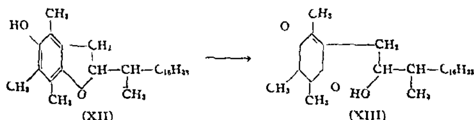
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P Karrer R F cher H Fritzsche H Keller B H Ringier and H Salomon *Helv Chim Acta* 21 131 (1938)

[45] P Karrer H Koenig B H *Ringier* and H Salomon *Ibid* 22, 1139 (1939)



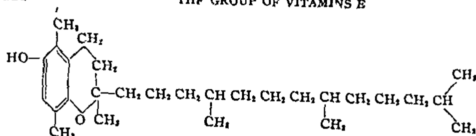

## (b) β Tocopherol

β Tocopherol has the empirical formula $C_{28}H_{48}O_2$ and thus differs from the empirical formula of α tocopherol only by one CH group Upon pyrolysis of the β compound, trimethyl hydroquinone[47, 48] (ψ cumoquinol) (II) resulted and upon cleavage with hydriodic acid p xylenol (III) was obtained Oxidation of β tocopherol with chromic acid yielded the same $C_{21}$ lactone (IV)[49] which was obtained from the α isomer β Tocopherol is thus the lower homolog of α tocopherol with only two methyl groups in the aromatic ring of the molecule These two methyl groups are in p position to each other β Tocopherol has therefore the structure (I) which was proved by synthesis

[47] W John *Z physiol Chem* 250 ı (1937) W John F Dietzel and P Günther *Ibid* 252 ı09 (1938) *Nat urwisse schaften* 26 366 (1938) W John *Z physiol Chem* 252 ?2? (1938)

[48] F Bergel A R Todd and T S Work *J Soc Chem I I* 56 10 ı4 (1937)

[49] O H Emerson *J Am Chem Soc* 60 1 41 (1938)



(I)

Pyrolysis → HI → CrO₃

(II) (III)

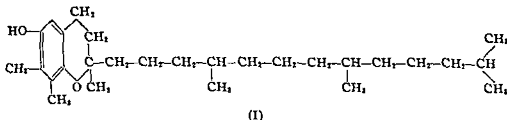
(IV)

One position in the benzene nucleus of β tocopherol is not substituted. This fact could be proved by the introduction of an allyl group by means of allyl bromide and zinc chloride.[50]

### (c) γ *Tocopherol*

γ Tocopherol has the empirical formula $C_{28}H_{48}O_2$ and is therefore an isomer of β tocopherol. Pyrolysis yields the same compound as does the pyrolysis of β tocopherol, namely, trimethyl hydroquinone. Oxidation with chromic acid yields dimethyl maleic anhydride.[51] γ Tocopherol is thus *o* xylo tocopherol (I).

[50] P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon, *Helv. Chim. Acta* 21, 939 (1938).
[51] O. H. Emerson and L. I. Smith, *J. Am. Chem. Soc.* 62, 1869 (1940).



(I)

## 7 Synthesis

The three known vitamins E, α β and γ tocopherol have been synthesized according to the same principle Alkylated hydroquinone is reacted with phytyl halide in the presence of a catalyst to form, in one reaction the desired chroman derivative Thus trimethyl hydroquinone (I) condenses with phytyl bromide (II) in benzene solution in the presence of zinc chloride to give in almost quantitative yield *dl* α tocopherol (III)[52] Instead of the phytyl halide phytol[53] or phytadiene[54] can be used successfully The condensation with any of these three phytyl compounds can be accomplished without catalysts The synthetic *dl* form (racemic about position 2 and perhaps about carbon atoms 4′ and 8′) can be resolved by esterification with bromo camphor sulfonic acid to yield an ester which appears to be identical with the naturally occurring α tocopherol[55, 56]

The syntheses of β and of γ tocopherol have been carried out by the same methods[57] Isomeric xylo hydroquinones have been reacted with phytyl compounds to yield the racemic β tocopherol (V) from *p* xylo hydroquinone (IV) and the racemic γ tocopherol (IX) from *o* xylo hydroquinone (VIII) A number of by products are obtained in these reactions by condensation of the hydroquinone with two molecules of the phytyl compound The yield of these by products is greater when zinc chloride is used as a catalyst than when formic acid is employed[58] The two types of by products which are obtained from for example *p* xylo hydroquinone are shown in formulas (VI) and (VII) Better yields of the tocols are also obtained when mono esters of the quinols for example the mono-ben

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[52] P Karrer H Fritzsche B H Ringier and H Salomon *Helv Chim Acta* 21 520 8 0 (1938) *Nature* 141 1057 (1937)

[53] F Bergel A M Copping A Jacob A R Todd and T S Work *Not re* 142 36 (1938) *J Chem Soc* 1938, 1382

[54] L I Smith H F Ungnade and W W Prichard *Science* 88 37 (1938)

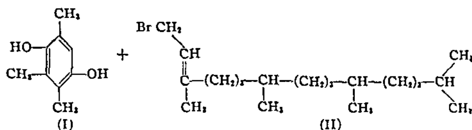
[55] P Karrer H Fritzsche B H Ringier and H Salomon *Helv Chim Acta* 21 520 8 0 (1938), *Nature* 141 1057 (1937)

[56] P Karrer H Koenig B H Ringier and H Salomon *Helv Chim Acta* 22 1139 (1939)

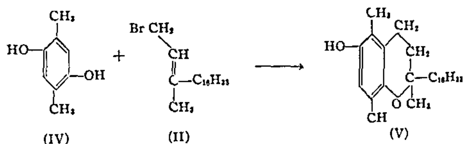
[57] P Karrer and H Fritzsche *Ibid* 21 1234 (1938) 22 ?60 (1939)

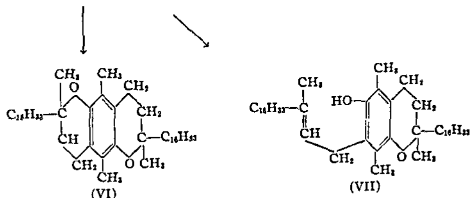
[58] P Karrer H Koenig B H Ringier and H Salomon *Ibid* 22 1139 (1939)

zoates, instead of the free quinols are used for the condensation [59] Subsequently the ester group is removed by hydrolysis

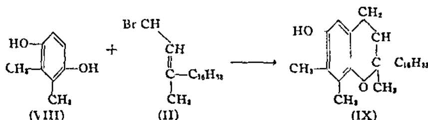



(III)

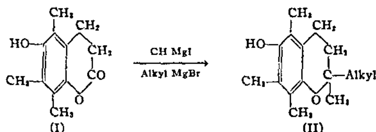


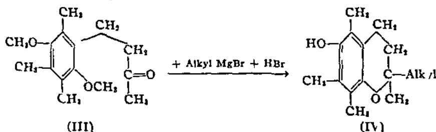

[59] A Jacob M Steiger and A R Todd *J Soc Chem Ind* 57 1188 (1938)




An entirely different type of synthesis should be mentioned which has no utility for the preparation of the tocopherols proper, but which has been used successfully for the preparation of similar compounds with different aliphatic side chains 5 7,8 Trimethyl 6 hydroxy 3 4 dihydro coumarin is condensed with a mixture of methyl-magnesium halide and alkyl magnesium halide to yield the desired tocopherol homolog [60]




Again a different type of synthesis, not well suited for the tocopherols themselves but attractive for certain of their homologs (IV) is the Grignard reaction of the ketone (III)[61 62] with an alkyl magnesium halide followed by treatment with hydrobromic acid



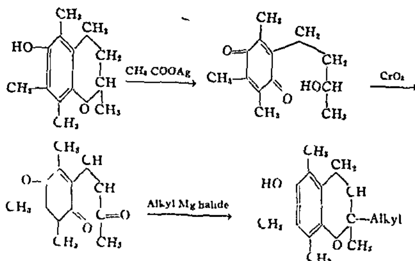

A last method should be mentioned which has been used for the preparation of tocol homologs This consists of the introduction of a second alkyl group in the 2 position of a 2 methyl 6-hydroxy chroman according to the reaction scheme [63]

[60] W John P Günther and M Schmeil *Ber* 71 263 (1938)

[61] W John and P Günther *Ibid* 72 1649 (1939)

[62] L I Smith H E Ungnade J W Opie W W Prichard R B Carlin and E W Kaiser *J Org Chem* 4 323 (1939)

[63] W John and M Schmeil *Ber* 72 1653 (1939)




## 8 Industrial Methods of Preparation

Vitamin E is marketed in the form of concentrates *from natural sources* and in the form of the racemic *dl* $\alpha$ tocopherol obtained by synthesis The latter is preferred by the medical profession The methods used for these processes are those which have already been described in the sections on the isolation and synthesis The synthetic tocopherol is sold in the form of the acetate which is at least as active as the free phenol and has the advantage of greater stability toward oxidation [64] The most important industrial method for the concentration of vitamins E from natural oils is the short path, high vacuum molecular distillation The natural vitamin F can be stabilized by the addition of antioxidants, such as hydroquinone or ascorbic acid [64]

## 9 Biogenesis

Nothing definite is known about the biogenesis of the vitamins E They are not synthesized in the animal organism, but in the plant It can be assumed that the synthesis in the plant cells is similar in principle to the laboratory methods for the synthesis of the tocopherols Phytol occurs in relatively large quantities in the plant, mainly in esterified form in chlorophyll but also in the free state as for example, in germ oils [65] There is some evidence for the assumption that vitamins E are synthesized preferentially in the green parts If this is true, the vitamin is transported into the seeds which usually contain higher amounts than, for example the leaves Of special interest is the observation that plant embryos for example, wheat

[64] O Isler *Helv Chim Acta* 21 1756 (1938)
[65] A R Todd F Bergel and T S Work *Biochem J* 31 22$_{}$07 (1937)

germs contain more vitamin E than the seed contained and more per unit weight than the plant will contain at any other stage of its life

The animal organism (for example rat) cannot synthesize vitamins E as has been stated before Such a synthesis *in vivo* does not occur, even when the starting materials of the laboratory synthesis namely phytol and trimethyl hydroquinone are fed to the animals [66]

## 10 Specificity

Synthetic *dl* α tocopherol has the same biological efficacy as the naturally occurring α tocopherol (2–3 mg correspond to one Rat Unit) The β and the γ isomers are only half as active as the α isomer (activity 5 mg ) whereas the *m* xylo tocopherol, which has not been found in nature, appears to be almost as active as the α tocopherol (activity 3 mg ) Most esters of the tocopherols show excellent vitamin E efficacy,[67, 68] with the exception of the allophanates which are completely inactive The acetate propionate and butyrate are said to be even more active than the free vitamin [68] The phosphoric acid ester of *dl* α tocopherol upon parenteral administration is more active than the vitamin itself [69] Etherification brings about a complete loss of activity

The tocopherol-quinones are not biologically active [70, 71, 72, 73] The difference in activity (in rats) of the naturally occurring vitamins E points toward a relatively great specificity of this vitamin This has been substantiated by the preparation and biological evaluation of a great number of compounds of more or less similar structure to the tocopherols Varying the substituents of the benzene ring it was found that 5,7 dimethyl 8-ethyl tocol[74, 75] and a diethyl methyl tocol[76] are active in a minimum dose of 10 mg while mono methyl tocol and 5 7 diethyl tocol are not active even in doses of 40–50 mg [77, 78] Tocol itself[78] and 6 desoxy tocol [79] at least up to 100 mg doses are also inactive

[66] H M Evans O H Emerson G A Emerson L I Smith H F Ungnade W W Prichard F L Austin H H Hoehn J W Opie and S Wawzonek *J Org Chem* 4 376 (1939)

[67] O Isler *Helv Chim Acta* 21 1756 (1938)

[68] V Demole O Isler B H Ringier H Salomon and P Karrer *Ibid* 22 65 (1939)

[69] P Karrer and G Bussmann *Ibid* 23 1137 (1940)

[70] P Karrer and A Geiger *Ibid* 23 455 (1940)

[71] W John E Dietzel and W Emte *Z physiol Chem* 257 180 (1939)

[72] P Karrer H Salomon and H Fritzsche *Helv Chim Acta* 21 309 (1938)

[73] M D Wright and J C Drummond *Biochem J* 34 32 (1940)

[74] P Karrer H Koenig B H Ringier and H Salomon *Helv Chim Acta* 22 1139 (1939)

[75] P Karrer and O Hoffmann *Ibid* 22 654 (1939)

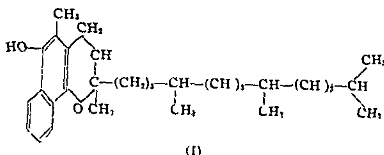
[76] P Karrer and O Hoffmann *Ibid* 23 1126 (1940)

[77] P Karrer and H Fritzsche *Ibid* 21 1234 (1938) 22 260 (1939)

[78] A. Jacob F K. Sutcliffe and A R Todd *J Chem Soc* 1940 327

[79] F v Werder T Moll and F Jung *Z physiol Chem* 257 129 (1939)

Of special interest is the naphtho tocopherol (I) which shows vitamin E activity in 25 mg doses, but also shows vitamin K activity *in doses between* 300 and 600 γ [80]




(I)

The introduction of a double bond into the chroman ring with the formation of a *dl* 3,4 dehydro α tocopherol has no significant influence on the activity, which is about 6 mg [81]

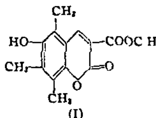
The structure of the long aliphatic side chain on the 2 position is of great *importance for the biological efficacy* Shortening this chain by one isoprene unit [82] that is by five carbon atoms or by two such units[83] causes loss of the activity (tested up to 40 mg) The same is true for similar substances which contain double bonds in the side chain, for example, for the compound prepared from trimethyl hydroquinone and farnesyl bromide [84] Compounds which have instead of the long aliphatic side chain only a methyl group or no side chain at all are also inactive [85] Activity in a 50–60 mg dose has however been claimed for a 2 dodecyl 2,5 7,8 tetra methyl 6 oxy chroman [86]

A number of other compounds which are less closely related chemically to the tocopherols have been investigated for vitamin E activity Surprisingly enough some have been found active at least in the rat test which is commonly employed for the determination of vitamins E All these other compounds, however, are active only in doses of an entirely *different order of magnitude* Thus several hydroquinones their esters and ethers have been found active *o* and *p* Xylo hydroquinone but not the *m* derivative are active in 100 mg doses [87] Trimethyl hydroquinone and durohydroquinone, but not *trimethyl ethyl hydroquinone*, are active at the same level A naphthoquinone namely, the 2 3 dimethyl 5 6 7 8

[80] M Tishler L F Fieser and N L Wendler *J Am Chem Soc* 62 1982 (1940)
[81] P Karrer R G Legler and G Schwab *Helv Chim Acta* 23 1132 (1940)
[82] P Karrer and K A Jensen *Ibid* 21 1622 (1938)
[83] P Karrer and K S Yap *Ibid* 23 581 (1940)
[84] P Karrer and K A Jensen *Ibid* 21 1622 (1938)
[85] P Karrer R Escher H Fritzsche H Keller B H Ringier and H Salomon *Ibid* 21 939 (1938)
[86] W John P Günther and M Schmeil *Ber* 71 2637 (1938)
[87] F v Werder T Moll and F Jung *Z physiol Chem* 257 129 (1939)

tetrahydro 1,4 naphthoquinone is reported to be active at high levels[85] A considerable number of esters and of ethers of trimethyl and of tetra methyl hydroquinone have been tested and practically all of them show some activity at high levels[87 88 89 90] The simple phenols and phenol ethers are inactive, but *o* allyl phenol di *o* hexenyl phenol and *p* amino *o* allyl phenol are active[91]

Chroman itself is active as are the 2,2 diethyl and 2,2 di *n* butyl derivatives but not the corresponding derivatives with odd numbers of carbon atoms 2 5,7,8 Tetramethyl chroman and 2 2,5,7,8 pentamethyl 6 hydroxy chroman are also active Quite a number of coumarins have been tested and were found to be inactive with the exception of compound (I) which is said to be active in doses as low as 20 mg 2 Methyl coumaran, 2 2 7 trimethyl coumaran and 2,3,4 6 7 pentamethyl 5 hydroxy coumaran show activity at about 100 mg doses



(I)

## 11 Determination

The only reliable method for evaluating vitamins E is the biological assay based on a comparison with the synthetic *dl* $\alpha$ tocopherol The physical and chemical methods have a number of shortcomings, the most important of which is that it is impossible to differentiate between the $\alpha$ $\beta$ and $\gamma$ isomers, which have different biological efficacies Furthermore the necessity of applying isolation procedures in order to separate interfer ing substances causes considerable loss

### (a) *Physical Methods*

**Spectroscopical Determination**[92 93]—The determination of vitamin E in alcohol or cyclohexane solution by its characteristic absorption spectrum

[88] F v Werder and T Moll *Ibid* 254 39 (1938)
[89] H M Evans O H Emerson and G A Emerson *Science* 88 193 (1938)
[90] E Fernholz and J Finkelstein *J Am Chem Soc* 60 402 (1938)
[91] H M Evans O H Emerson G A Emerson L I Smith H E Ungnade W W Prichard F I Austin H H Hoehn J W Opie and S Wawzonek *J Org Chem* 4 376 (1939)
[92] T Moore and K R Rajagopal *Biochem J* 34 335 (1940)
[93] F J Cuthbertson R R Ridgeway and J C Drummond *Ibid* 34 34 (1940)

with a maximum of 294 mμ can only be recommended for solutions of the pure or almost pure compounds The naturally occurring substances with vitamin E activity comprise besides α tocopherol, its esters, which have different absorption maxima and less pronounced extinction coefficients, for example, α tocopherol, $E^{1\%}_{1\,cm}$, 284 mμ = about 77 α tocopherol acetate, $E^{1\%}_{1\,cm}$, 285 mμ = about 42 Furthermore, even traces of vitamin A interfere due to the strong absorption characteristics [$E^{1\%}_{1\,cm}$, 328 mμ = 1725]

Natural materials such as plant oils and the fat extract of animal materials contain many other substances which have no vitamin E activity but exhibit absorption in the region of the vitamin E absorption spectrum and interfere therefore with the spectroscopical determination of the vitamin Furthermore, any extraction of the vitamin from animal tissues causes some loss In order to obtain any data it is necessary to saponify the material, for example, the fat extract, germ oil, etc The saponification destroys further amounts of the vitamin,[94] unless it is carried out under most carefully controlled conditions [95]

A modification of the spectroscopical determination of the tocopherols consists in the spectroscopical determination of the oxidation product of the vitamin [96] The vitamin is best oxidized[97] with a 5% solution of silver nitrate in 90% methyl alcohol The quinone formed shows an absorption maximum at 265 mμ with an extinction coefficient about four times as high as that of the vitamin itself Spectroscopical assays are run before and after the oxidation of the vitamin to exclude the values obtained from any previously oxidized material

## (b) *Chemical Methods*

**Ferric Chloride-Dipyridyl Method** [98]—This method is based on the oxidation of vitamin E in alcoholic solution by ferric chloride [99] An addition of α,α′ dipyridyl develops a characteristic red color with the resulting ferrous chloride A blank is run with each determination Working in subdued light is recommended, since sunlight tends to cause a development of color in the blank solution

[94] A Emmerie and C Engel *Rec trav chim* 58 895 (1939)
[95] A Emmerie *Ibid* 59 246 (1940)
[96] W John *Soc Chem Ind Food Group* 1939 73
[97] W I J Cuthbertson R R Ridgeway and J C Drummond *Biochem J* 34 34 (1940)
[98] A Emmerie and C Engel *Nature* 142 873 (1938) *Rec trav chim* 57 13 1 (1938)
[99] J Waddell and H Steenbock *J Biol Chem* 80 431 (1928)

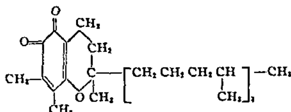
This method is not very specific since the color is given by all reducing compounds. Other substances such as the carotenoids interfere by obscuring the color of the ferrous dipyridyl. Although it is possible to estimate the vitamin E content of unsaponified oils, these oils often contain other reducing agents which can be removed by saponification. The best results are obtained by hydrolysis with 2 *N* KOH methanol solution for 10 minutes.[100] It has, furthermore, been suggested that all interfering substances be adsorbed on, for example, floridin which has been activated by treatment with hydrochloric acid.[101]

A modification of this procedure consists[102] in treating the material to be assayed in a hexane or petroleum ether solution with 85% sulfuric acid followed by centrifuging and washing with dilute alkali. The interfering substances are largely removed and the vitamin E is determined in the remaining solution by ferric chloride and dipyridyl.

**Gold Chloride Method.**[103, 104]—This method consists in the oxidation of vitamin E with gold chloride at about 50° C. The progress of the reaction is followed electrometrically. The equilibrium is but slowly established.

The accuracy of this method, like that of the ferric chloride dipyridyl method, is affected by other reducing substances, including carotenoids. For accurate determinations this method requires larger quantities of the vitamin than needed in the ferric chloride dipyridyl method.

**Nitric Acid Method.**[105]—Vitamin E, upon heating with alcoholic nitric acid, yields a characteristic red color. The coloration is caused by the production of the following compound:[106]



Since other naturally occurring substances give similar, or at least yellow colors, the use of a filter limited to the wave band 450–520 mμ has been

[100] A. Emmerie: *Rec. trav. chim.* 59, 246 (1940).
A. Emmerie and C. Engel: *Ibid.* 58, 283 (1939).
[102] W. E. Parker and W. D. McFarlane: *Can. J. Research* 18, 405 (1940).
P. Karrer, R. Escher, H. Fritzsche, H. Keller, B. H. Ringier and H. Salomon: *Helv. Chem. Acta* 21, 939 (1938).
[104] P. Karrer and H. Keller: *Ibid.* 21, 1161 (1938).
[105] M. Furter and R. E. Meyer: *Ibid.* 22, 240 (1939).
[106] L. I. Smith, W. B. Irvin and H. E. Ungnade: *J. Am. Chem. Soc.* 61, 2424 (1939); *Science* 90, 334 (1939).

advocated The color is then measured in one of the common types of colorimeters

A distinct disadvantage of this method is the fact that the simple biologically inactive oxidation product of the tocopherols gives the same coloration with nitric acid Thus, vitamin E destroyed, for example, by air oxidation or by ferric chloride, shows up in this test as being present as vitamin E On the other hand, this method has the advantage that carotenoids do not interfere Appropriately used, this assay procedure is very convenient and rapid [107]

### (c) Biological Methods

**Fertility Test on Rodents** [108, 109] For biological determinations of the vitamin E, female animals (rats) are usually preferred, since they can be essentially healed several times from the avitaminosis, whereas in males (rats) the damage done by the absence of the vitamin is irreparable Rats are used usually in these tests, although occasionally other animals, for example, the rabbit,[110] have been recommended Female virgin rats preferably are used[111, 112] (at about 30 to 40 days after weaning), since the initial storage of the vitamin in the young can be controlled through placental and mammary transfer

The tests can be carried out by prophylactic or by curative methods, but the latter is considered much more reliable The criterion is the proof of the occurrence of gestation and resorption of the fetuses This can be accomplished by following the weight increase and decrease, respectively, after mating Conception is proved by the plug, that is the bouchon vaginale Successful implantation of the ova can be verified by the placental sign, that is detection of blood in the vagina between the ninth and thirteenth days after mating The percentage fertility is measured in this test An improved method which is based on a graded response of the animals consists in the autopsy of all animals at the tenth day of pregnancy The evaluation comprises a determination of the weight of the uterus, both before and after removal of living fetuses, dead fetuses and resorption sites The results of this latter method are expressed in the so called uterine index '

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[107] H E Ungnade and L I Smith *J Org Chem* 4 337 (1939)

[108] H M Evans and G O Burr *Proc Natl Acad Sci U S* 11 334 (1925) H S Olcott and H A Mattill *J Biol Chem* 104 423 (1934)

[109] H M Evans and G O Burr *Mem Univ Calif* No 8 (192 )

[110] C G Mackenzie and E V McCollum *J Nutrition* 19 345 (1940)

[111] A L Bacharach E Allchorne and H E Glynn *Biochem J* 31 2287 (1937)

[112] A L Bacharach and E Allchorne *Ibid* 32 1298 (1938)

**Fertility Test on Daphnia** The use of *Daphnia magna* the trans parent crustacea as a test animal has been suggested for the quantitative estimation of vitamin E[113] The criterion in this assay procedure is the growth of the ovaries and of the parthenogenic embryos in the brood sac

## 12 Standards

Synthetic racemic alpha tocopherol acetate has been adopted as the international standard for vitamin E and it has been recommended that the International Unit for vitamin E be defined as the specific activity of 1 mg of the standard preparation This quantity is the average amount which when administered orally, prevents resorption gestation in rats deprived of vitamin E[114] The international standard is issued in the form of a solution in olive oil of which one International Unit is contained in 0 1 g

One Rat Unit or the fertility dose is the smallest amount of vitamin E which when given *per os* daily to resorption sterile female rats for the entire period of gestation (21 days) brings about in 50% of the animals the birth of at least one living young

One Rat Unit or the fertility dose is equal to 2–3 mg of $\alpha$ tocopherol

The Pacini Linn Unit is equal to $^{1}/_{10}$ of the fertility dose

The Bomskow Rat Unit[115] is the amount of vitamin E which when ad ministered once during the first eight days of pregnancy prevents resorp tion

## 13 Physiology of Plants and Microorganisms

The present day knowledge of the significance of the occurrence of vita mins E in plants is very meager It must be assumed that the tocopherols play a very definite role in the plant cells and that this role is not primarily concerned with the antioxidative properties of the compounds It is probably quite significant that the seeds usually contain higher amounts than any other part of the living plant On the other hand the germs for example wheat germs contain even higher amounts The possibility of an influence upon cell growth must therefore be considered

Not all organisms contain tocopherols The microorganism *Phycomyces*, for example does not seem to contain any at all as evident from electro metric titration with gold chloride[116]

[113] A Viehoever and I Cohen *Am J Pharm* 110 29 (1938) A Viehoever *J Assoc Official Ag Chem* 22 715 (1939)

[114] E M Hume *Nature* 148 47 (1941)

[115] C Bomskow *Arch exptl Path Pharmakol* 190 [illegible] (1938)

[116] W H Schopfer and S Blumer *Z Vitaminforsch* 9 344 (1939)

Vitamin E when injected into higher plants in olive oil solution exerts an inhibitory effect upon the growth (experiments with *Melandrium album* (Miller) Garke) [116]

## 14 Animal Physiology

### (*a*) *Metabolism of Vitamins E*

Vitamins E and their esters are easily absorbed from the intestinal tract especially in the presence of bile acids [117] The vitamin is, however not well utilized when given parenterally [118] The vitamin is taken up by the blood in which its presence can be demonstrated The blood of female rats contains approximately 5 6 γ and that of male rats 6 4 γ of tocopherols per 10 cc of serum [119] The blood level of rats can be increased up to 100 γ by oral administration of the vitamin Upon an intake of esters for example the acetate, the free vitamin appears in the blood

No significant excretion of vitamin E is observed [120] as long as normal doses of the vitamin are fed The intake of excess doses causes the excretion of a certain amount in the feces,[119, 121] but only traces are found in the urine [119] This means that the vitamin is inactivated in the organism probably by an oxidation mechanism Since, furthermore no simple oxidation products of the tocopherols are excreted, the vitamin molecule must be broken down in the organism (rats) [119]

Vitamins E are stored to a small but significant extent in animal body fats [119, 122] in the muscles,[119, 121] and especially high amounts are found in the placenta and in the anterior lobe of the pituitary gland Vitamin E is secreted in the milk,[123] and in the eggs of birds

The liver of horse and cattle contains unusually high amounts,[124] but no vitamin E is stored in the liver of rats even on excessive dosage of the vitamin [119, 125]

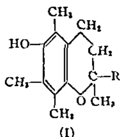
### (*b*) *Physiological Action of Vitamins E*

Much work and much speculation concerning the physiological action of the vitamins E arose when the constitution of the vitamins became known

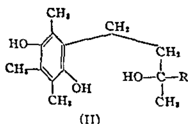
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117 J D Greaves and C L A Schmidt *Proc Soc Exptl Biol Med* 37 40 (1937)
118 M Goettsch and A M Pappenheimer *J Nutrition* 22 463 (1941)
119 A Emmerie and C Engel *Rec trav chim* 58 895 (1939) W F J Cuthbertson R R Ridgeway and J C Drummond *Biochem J* 34 34 (1940)
120 C S McArthur and E M Watson *Can Chem Proc Ind* 23 350 (1939)
121 A Juhasz Schäffer *Arch path Anat (Virchow's)* 281 53 (1931)
122 H M Evans and G O Burr *Mem Univ Calif* No 8 (1927)
123 M M O Brien *Biochem J* 32 1474 (1938)
124 P Karrer W Jaeger and H Keller *Helv Chim Acta* 23 464 (1940)
125 T Moore A J P Martin and K R Rajagopal *Soc Chem Ind Food Group* 1939 11

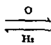
The fact that they are phenols and that they are antioxidants led to the supposition that vitamins E may take part in some oxidation reduction mechanism From a chemical standpoint it would be conceivable to assume that the vitamin acts in the following manner

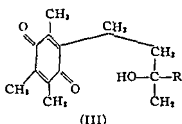


(I)



(II)



(III)

No support for this theory can however be offered The quinone (III) which can be obtained by oxidation of the vitamin is completely inactive biologically [126, 127 128 129] Thus a reduction to the quinol (II) in the cells seems quite improbable

The many symptoms which can be observed during a deficiency of vitamin E points to the assumption that vitamin E is of importance to the tissues as a whole Unconfirmed experimental findings to the effect that the organism of vitamin E depleted animals contains unsaponifiable material which upon injection into rats causes the symptoms of a vitamin E deficiency, have tentatively been interpreted as indicating a disturbance of some metabolic processes perhaps of the fat metabolism [130]

A possible relation of vitamin E to the amino acid metabolism is seen in the fact that during avitaminosis the creatine content of striated muscles is

[126] P Karrer and A Geiger *Helv Chim Acta* 23 455 (1940)
[127] W John E Dietzel and W Emte *Z physiol Chem* 257 180 (1939)
[128] P Karrer H Salomon and H Fritzsche *Helv Chim Acta* 21 309 (1938)
[129] M D Wright and J C Drummond *Biochem J* 34 32 (1940)
[130] B A Kudrjashov *Bull biol et med exper URSS* 3 279 (1937)

greatly reduced[131] in rabbits[132] and in rats[133] and that the urinary excretion of creatine is considerably increased[13, 134] while the excretion of creat r ne remains unchanged A marked reduction of the creatine content in the urine is observed following the administration of vitamin E No such changes have, however, been found in the urine of man suffering from progressive muscular dystrophy amyotonia congenita and similar neuro muscular disturbances [135]

The primary physiological action of vitamin E is apparently to direct certain activities of the cell nucleus [136, 137] This conception of the mecha nism of the vitamin E action, although much disputed has proved to be of extreme value in an attempt to correlate the various experimental facts and observations of the many different symptoms of vitamin E de ficiency

The role which vitamin E plays in the metabolic activities of the cell nucleus cannot as yet be defined exactly It seems that the vitamin function is more intimately concerned with the processes of cell maturation and differentiation than with mitosis Thus the rate of growth of the Walker 256 mammary carcinoma is not essentially affected by continuous mainte nance in vitamin E deficient rats [138] On the other hand, an increase in growth has been demonstrated in *in vitro* experiments with liver spleen heart and periosteal tissue cultures upon addition of vitamin E [139] Re tarded growth has as in the case of deficiencies of the other vitamins also been observed during vitamin E deficiency [140] The considerable decrease in the weight of the testis even when calculated in relation to the reduced body weight is however, specific for this vitamin [141] In accordance with the conception that vitamin E is of special influence to all those tissues in which cellular proliferation and differentiation proceed at a high speed it has been found that the application of this vitamin is especially useful for example to effect growth of prematurely born babies or to heal skin wounds in rats [14, 143] Beneficial effects in the latter case have been ob served upon oral or local application

131 M Goettsch and E F Brown *J Biol Chem* 97 549 (1932)
132 C G Mackenzie and E V McCollum *J Nutrition* 19 345 (1940)
133 F Verzár *Schweiz med Wochschr* 69 738 (1939)
134 S Morgulis and H C Spencer *J Nutrition* 11 573 (1936)
135 W Fleischmann *Proc Soc Exptl Biol Med* 46 94 (1941)
136 A Juhasz Schäffer *Arch path Anat (Virchow s)* 281 3 (1931)
137 K E Mason *Am J Anat* 52 153 (1933)
138 K E Mason *Soc Chem Ind Food Group* 1939 31
139 A Juhasz Schäffer *Arch path Anat (Virchow s)* 281 35 (1931)
140 H Blumberg *J Biol Chem* 108 227 (1935)
141 A M Copping and V Korenschevsky *Soc Chem Ind Food Group* 1939 44
142 G Léranth and L Frank *Orvosi Hetilap* 80 778 (1936) *Chem Abstracts* 30 7634 (1936)
143 F R Adamstone *J Morphol* 52 47 (1931)

The histopathological changes which have been observed to occur in the testes of vitamin E depleted rats are largely responsible for the conception of the function of vitamin E The chromatin of the spermatozoa undergoes a typical change, a lysis and the nuclei become crescentric Upon further development of the deficiency disease the mature and immature cells the permatogonia, spermatocytes and spermatozoa fuse into giant cells with many nuclei Besides chromatolysis there appears to be an interference in the formation of chromatin

Similar effects are observed in the fetus The earliest signs of a vitamin E deficiency in the embryo is observed in the hematopoietic tissue the growth of which is disturbed The mesodermal tissues where rapid cellular activity prevails are similarly affected The symptoms of vitamin E deficiency to be discussed in the next section namely nervous lesions and degenerative lesions in the muscles seem to be related to the cerebral cortex (observations on rats) a tissue of high cellular activity

All experimental evidences lead to the conclusion that secondary to the lesions in the gonads and in the neuro muscular system and probably as the result of these rather than as their cause abnormalities occur in the pituitary and thyroid glands In young vitamin E depleted rats a change in the fur has been observed which is considered characteristic for hypophysectomized animals [144] [145] In the anterior lobe the acidophil and basophil cells degenerate and show no distinct granulation [146] The histological appearance of the anterior lobe is quite similar to the degeneration observed in the case of castrated animals Actually many of the symptoms observed as the result of a vitamin E deficiency resemble very closely the syndromes of a hypophysectomy [147] [148] The changes observed in the testes are however dissimilar [149] Vitamin E deficiency causes a degeneration of the germinative tissue whereas upon dysfunction of the pituitary the interstitial tissue is degenerated In male rats deprived of vitamin E the pituitaries were found to contain a greater gonadotropic action than found in the glands of rats fed with vitamin E [150] [151] In female rats deprived of *vitamin E the pituitary contains a decreased amount of luteinizing hormone* [152] Nevertheless the important symptoms of vitamin E deficiency, namely muscular dystrophy gestation resorption and testicular degenera

[144] F Verzár *Abderhaldens Handb biol Arbeitsmeth den* Abt 5 Pt 36 No 8 1269 (1937)
[145] F Verzár and E Kokas *Arch ges Physiol (Pflügers)* 227 511 (1931)
[146] M M O Barrie *Lancet* II 51 (1937)
[147] M M O Barrie *J Soc Chem Ind* 55 1063 (1936)
[148] F Verzár *Arch ges Physiol* 227 499 (1931)
[149] K E Mason *Am J Anat* 52 153 (1933)
[150] W O Nelson *Anat Record* 56 241 (1933)
[151] J C Drummond *Soc Chem Ind Food Group* 1939 27
[152] I W Rowlands and F Singer *J Physiol* 86 323 (1936)

ture which has been found in rats,[170, 171, 172, 173, 174, 175] rabbits,[176, 177, 178] dogs,[179] guinea pigs[180, 181, 182, 183] and chicks.[184] Before the overt symptoms of such a dystrophy appear, the following syndromes can be detected:[185] increase in the concentration of water and of chlorides in the muscles, decrease in the maximum strength and focal hyaline necrosis of muscle fibers. These lesions are accompanied by degenerative changes in the central nervous system,[186] histologically evident by degeneration of nerve cells in the spinal cord.[187] In cases of severe avitaminosis, paralysis first of the hind and then also of the fore limbs is found in young[188] and in old rats. Carpopedal spasms and convulsions have been observed in the final stages when the mortality is 100%.[189]

Another pathological change observed in rats deprived of vitamin E is a considerable enlargement of the thymus both in male and in female animals.[190] The changes which occur in the pituitary, the thyroids and the kidney have already been discussed. In the young vitamin E deficient rat also an uncalcified skull has been observed.

In beef cattle and in milk cows, sterility and early abortion have successfully been treated with vitamin E and the existing evidence leads to the conclusions that some of these animals suffered from a true E avitamino

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170 See however M. Goettsch and J. Ritzmann, *J. Nutrition* 17, 371 (1939).

171 H. M. Evans and G. O. Burr, *J. Biol. Chem.* 76, 273 (1938). H. Blumberg, *Ibid.* 108, 227 (1935). G. O. Burr, W. R. Brown and R. L. Mosely, *Proc. Exptl. Biol. Med.* 36, 780 (1937). A. Ringstead, *Biochem. J.* 29, 788 (1935).

172 H. S. Olcott, *J. Nutrition* 15, 221 (1938).

173 H. M. Evans and G. O. Burr, *J. Biol. Chem.* 76, 273 (1928).

174 L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen, 1938.

175 M. D. Lipshutz, *Rev. neurol.* 65, 221 (1936). J. Morelle, *Compt. rend. soc. biol.* 108, 804 (1931). H. S. Olcott and H. A. Matill, *J. Biol. Chem.* 104, 423 (1934).

176 C. G. Mackenzie and E. V. McCollum, *J. Nutrition* 19, 345 (1940).

177 S. Morgulis, *Monographie Actualités Scientifiques et Industrielles*, Paris, 1938, p. 74.

178 S. G. Morris, *Science* 90, 424 (1939).

179 H. D. Anderson, C. A. Elvehjem and J. E. Gonce, *Proc. Soc. Exptl. Biol. Med.* 42, 750 (1939). K. M. Brinkhous and E. D. Warner, *Am. J. Path.* 17, 81 (1941).

180 M. Goettsch and A. M. Pappenheimer, *J. Exptl. Med.* 54, 145 (1931).

181 H. S. Olcott, *J. Nutrition* 15, 221 (1938).

182 N. Shimotori, G. A. Emerson and H. M. Evans, *Science* 90, 89 (1939). *J. Nutrition* 19, 547 (1940).

183 E. L. Wood and H. M. Hines, *Proc. Soc. Exptl. Biol. Med.* 36, 786 (1937).

184 H. Dam and J. Glavind, *Naturwissenschaften* 28, 207 (1940). *Nature* 143, 810 (1939).

185 G. C. Knowlton and H. M. Hines, *Proc. Soc. Exptl. Biol. Med.* 38, 655 (1938); 42, 133, 804 (1939).

186 D. Lipshutz, *Rev. neurol.* 65, 221 (1936). L. Einarson and A. Ringstead, *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats*, Copenhagen, 1938.

187 M. Ekblad and G. Wohlfart, *Z. ges. Neurol. Psychiat.* 168, 144 (1940).

188 H. M. Evans and G. O. Burr, *J. Biol. Chem.* 76, 273 (19 8).

189 M. M. O. Barrie, *Lancet* II, 251 (1937).

190 A. M. Copping and V. Korenschevsky, *Soc. Chem. Ind. Food Group* 1939, 44.

sis[191, 192] There is also considerable evidence[193] that administration of vitamin E is of value in the treatment of the infectious abortion in cows, caused by the *Bacillus abortus* (Bang) probably due to the fact that vitamin E *increases the resistance of the animals against infection*[194]

In sows barrenness has also successfully been overcome by vitamin E therapy[195]

The pathological changes observed on rats are essentially similar to the changes observed in birds Eggs of hens reared on a vitamin E deficient diet cannot be hatched successfully In the embryos a lethal ring develops in the blastoderm from cell proliferation in the mesoderm The organism dies from starvation and hemorrhages after the blood vessels of the blastoderm are choked off[196] Hatchability is greatly improved upon adding vitamin E to the chicken feed[197, 198, 199] In the male fowl degeneration of the testis has been observed[198, 197] In chicks a vitamin E deficiency causes *alimentary exudative diathesis followed by increased capillary permeability and muscular dystrophy*[200] Nutritional encephalomalacia of the chick is caused by vitamin E deficiency[201]

The question as to whether or not an E avitaminosis occurs in man has not definitely been decided Considerable evidence exists for the fact that vitamin E may exert a beneficial influence in certain cases of habitual abortion[202, 203] and possibly also of threatened abortion and premature separation of the placenta Certain forms of toxemia in pregnancy[204] have also been reported to respond favorably to a treatment with vitamin E It has furthermore been stated that administration of vitamin E is beneficial

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[191] F Vogt Möller and F Bay *Münch tierärztl Wochsch* 82 637 (1931) *Vet J* 87 165 (1931) 90 288 (1934)

[192] A Andersen *Medlemsblad dansk Dyrlaeg foren* 17 113 (1934) H Lehmke *Berlin tierärztl Wochschr* 1938 367 J P Tutt *Vet J* 89 416 (1933)

[193] R Moussu *Compt rend* 201 12 8 (1935)

[194] A E Lange *Tierärztl Rundz* 1938 239

[195] R W Lentz *Berl u Münch tierärztl Wochschr* 1938 201

[196] F R Adamstone *J Morphol* 52 47 (1931)

[197] L F Card *Poultry Sci* 8 3 8 (1929)

[198] G I Barnum *J Nutrition* 9 621 (1935)

[199] F Ender *Z Vitaminforsch* 4 106 (1935) see however C E Holmes and W W Cravens *Poultry Sci* 19 303 (1940)

[200] H Dam and J Glavind *Naturwissenschaften* 28 207 (1940) *Nature* 143 810 (1939)

[201] A M Pappenheimer M Goettsch and E Jungherr *Connecticut Agric Exp Stat* Bull. 229 (1939)

[202] P Vogt Möller *Lancet* 221 18? (1931) *Acta Obstet Gyn Scand* 13 219 (1933) *Klin Wochschr* 15 1883 (1936)

[203] D W Currie *Brit J Med* II 1218 (1937) J Gierhake *Arch Gynäkol* 156 348 (1933) A Juhasz-Schäffer *Ergeb inn Med* 45 129 (1933) H Martius *Med Welt* 1937 407 E V Shute *J Obstet Gynaecol Brit Empire* 42 10 1 1085 (193 ) 43 4 (1936) *Am J Obstet Gynecol* 33 429 (193 ) F M Watson *Canad Med Assoc J* 34 134 (1936) F M Watson and W P Tew *Am J Obstet Gynecol* 31 25? (193 ) J Young *Brit Med J* I 953 (1937)

[204] F V Shute *Am J Obstet Gynecol* 33 4?9 (1937) J Young *Brit Med J* I [illegible]

in cases of muscular dystrophy[205, 206] amyotrophic lateral sclerosis[205, 207, 208] myelopathy from pernicious anemia, neuromuscular syndromes *roaring* sensations in the ears, anorexia[209] and primary fibrositis[210] The information gained from these reports must, however, be regarded as provisional and all clinical use of vitamin E is still considered purely experimental

### (a) *Clinical Test Methods*

**Determination of Vitamin E in Urine** *Urinary Creatine Determination*[211]—Vitamin E hypovitaminosis can be detected by urine analysis since the creatine output increases rapidly *In rabbits for example, the* daily creatine excretion rises from a normal level of less than 10 mg to over 20 mg By the time symptoms of muscular dystrophy occur, 50 to 150 mg of creatine are excreted[21] This method is only of restricted value since many other factors may influence the creatine output

**Determination of Vitamin E in Blood** *The Ferric Chloride Dipyridyl Method* For a successful determination of vitamins E in blood it is necessary to extract the active material This can, for example be accomplished by treating the serum with dilute alkali in the cold and in the presence of formaldehyde and ethyl alcohol The vitamin is then extracted with ether and the ether solution is washed neutral with alkali and acid solutions By selective adsorption, for example, on floridin,[213] the carotenoids are removed The determination of the vitamin E in the remaining solution is then carried out according to the ferric chloride dipyridyl method As little as 5 cc of blood can be used for this method This method fails to be accurate for amounts of vitamin E less than 5 $\gamma$

## 16 Hypervitaminosis

Vitamins *E* are non toxic substances *E hypervitaminosis is unknown* Synthetic *dl* $\alpha$ tocopherol causes no toxic symptoms in rats even when given orally in doses as high as 50 g per kilogram of body weight The administration of several grams of vitamin E daily over a period of one to

[205] F Bicknell *Lancet* I 10 (1940)
[206] S Stone *J Am Med Assoc* 114 2187 (1940)
[207] I S Wechsler *Ibid* 114 948 (1940) T D Spies and R W Vilter *Southern Med J* 33 663 (1940)
[208] L Einarson and A Ringstead *Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats* Copenhagen 1938.
[209] T D Spies D P Hightower and L H Hubbard *J Am Med Assoc* 115 29? (1940)
[210] C L Steinberg *Am J Med Sci* 3 347 (1941)
[211] O Folin *J Biol Chem* 17 469 (1914)
[212] C G Mackenzie and F V McCollum *J Nutrition* 19 34 (1940)
[213] A Emmerie and C Engel *Rec trav chim* 58 283 (1939)

two months does not result in any adverse symptoms in rats dogs and cats The functions of the kidneys, the intestines the nervous system, the muscles and the sex organs are not affected by such doses [214]

## 17 Requirements

It has been proved experimentally that vitamin E is necessary for mice rats rabbits guinea pigs dogs chicks and ducks The crustacea Daphnia also needs vitamin E [215] Goats can apparently get along without an external supply of this vitamin [216] The requirements for man are largely unknown as stressed before

The vitamin E requirement of the rat is different for the male and the female animal It seems that the male animal needs only about one seventh to one tenth of the amount required by the female A similar but perhaps more marked difference is observed in mice

The average daily need of a female rat is from 2-3 mg of $\alpha$ tocopherol The minimum fertility requirements of female rats are 15 mg per kilogram body weight daily For rabbits the daily antimuscular dystrophy dose is about 1 mg of $\alpha$ tocopherol per kilogram body weight [217] In clinical therapy a dose of 6 mg of $\alpha$ tocopherol per day has been recommended for women [218]

[214] V Demole *Z Vitaminforsch* 8 338 (1939)
[215] A Vichoever and I Cohen *Am J Pharm* 110 297 (1938)
[216] G K L Underbjerg *Iowa State College J Sci* 15 107 (1940)
[217] C G Mackenzie and E V McCollum *J Nutrition* 19 345 (1940)
[218] D W Currie *Soc Chem Ind Food Group* 1939 77

# VITAMIN H—
# BIOTIN

# VITAMIN H—BIOTIN

## 1 Nomenclature

**Names**

Bios II (Lucas 1924)
Factor X (Boas 1927[1])
Vitamin H (Gyorgy 1931[2])
Coenzyme R (Allison Hoover and Burk 1933[3])
Bios II *b* (Miller 1934)
Anti egg white injury factor (Lease and Parsons 1934[4])
Biotin (Kögl 1935[5])
Factor W (Elvehjem 1936[6])
Vitamin $B_w$ (Lunde and Kringstad 1940[7])
S or Skin Factor (Marshall 1939[8, 9])

**Empirical formula**


$C_{10}H_{16}O_3N_2S$

**Efficacy**

One gram of biotin methyl ester = 27 000 000 Rat Units
= 25 000 000 000 Saccharomycetes Units

## 2 Chronology

1901 WILDIERS recognized that yeast needs a special material bios in addition to the known nutrients for optimal growth This had already been postulated by LIEBIG in 1869

1916 BATEMAN[10] made the casual observation that egg white exhibits a definite toxicity

1923 FULMER discovered the multiple nature of bios

1924 LUCAS separated bios into two components bios I and II

1927 BOAS[11] found that certain foods contain an organic substance which protects against egg white toxicity

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[1] M A. Boas *Biochem J* 21 712 (1927)
[2] P György *Z a sll Fortbild ng* 28 377 417 (1931)
[3] F E All son S R Hoover and D Burk *Sc nce* 78 217 (1933)
[4] J G Lease and H T Parson *Bioch n J* 28 2109 (1934)
[5] F Kögl *Ber* 68 16 (1935)
[6] C A Elvehjem C J Koehn and J F Ol son *J B ol Chem* 115 707 (1936)
[7] G Lund nd H Kring t d *J N t it on* 19 3 1 (1940)
[8] W Marsh ll *J I v st De matol* 2 205 (1939)
[9] W Marsh ll *Med W o ld* 57 101 (1939)
[10] W G B teman *J Biol Chem* 26 63 (1916)
[11] M A Boa *Biochem J* 21 71 (1927)

1928 EASTCOTT[12] isolated bios I in the pure form and identified it with *i* inositol
1931 GYÖRGY[2] recognized the necessity of an anti egg white injury factor (vitamin H) for man
1933 MILLER showed that bios II contained at least two different factors
1935 KÖGL[13] isolated biotin one of the compounds of bios II in the pure form
1940 GYÖRGY MELVILLE BURK and DU VIGNEAUD[14] identified vitamin H with biotin
1942 DU VIGNEAUD HOFMANN and MELVILLE suggested structural formulas for biotin on the basis of degradation reactions[14]

## 3 Occurrence

Vitamin H occurs in small amounts in all higher animals (mammals and birds) The only tissue found so far which is apparently devoid of this vitamin is the lens of the eye[15] The highest concentrations have been observed in liver[1, 16] kidney[1] and eggs[15] while milk[16] contains this vitamin in somewhat smaller concentrations

Vitamin H is found widely distributed in the plant kingdom Vegetables grains, nuts, etc contain considerable quantities[16] Most strains of yeast contain some vitamin H[16] In plants the tips of the roots and of the coleoptiles usually have a somewhat higher concentration than the rest of the plant[17] Relatively high concentrations have been observed in seeds and in pollen[17]

Vitamin H occurs predominantly in the free state in fruits and in grasses In grains, nuts and vegetables this vitamin is present partly in a bound form and partly free[18] In yeast and in animal tissues for example, in liver, vitamin H occurs mainly as a chemically bound compound

## 4 Isolation

As pointed out previously vitamin H occurs in many sources chemically bound and cannot be dissolved by any solvent In order to liberate the vitamin, for example, from yeast,[19] the material is autolyzed from four to six days Liver, on the other hand does not possess the necessary enzyme system for the autolytic liberation of this vitamin[20] Vitamin H can how

[12] E V Eastcott *J Phys Chem* 32 1094 (1928)
[13] F Kögl *Ber* 68 16 (1935)
[14] P György D B Melville D Burk and V du Vigneaud *Science* 91 243 (1940)
[14] V du Vigneaud K Hofmann and D B Melville *J Am Chem Soc* 64 188 (1942)
[15] F Kögl and W van Hasselt *Z physiol Chem* 243 189 (1936)
[16] C Lunde and H Kringstad *J Nutrition* 19 321 (1940)
[17] F Kögl and A J Haagen Smith *Z physiol Chem* 243 209 (1936)
[18] J O Lampen A A Kline and W H Peterson *Proc Am Soc Biol Chem* 1941 LXXIV J O Lampen G P Bahler and W H Peterson *J Nutrition* 23 11 (1942)
[19] P György *J Biol Chem* 131 733 (1939)
[20] P György R Kuhn and E Lederer *Ibid* 131 745 (1939)

ever be liberated by proteolytic digestion of liver[20 21 22 23] or by hydrolysis with acids,[21] for example, with $2N$ mineral acids with or without pressure [20] In contrast to fresh liver, the vitamin can be liberated from liver powder by hydrolysis at high pressure without addition of acids After the hydrolysis has been achieved according to any of these procedures the solution is filtered and impurities may be removed from the filtrate by precipitation with alcohols or lead acetate The vitamin can be dissolved in acetone from a concentrated filtrate whereby considerable amounts of impurities separate Further concentration has been achieved by precipitation with phosphotungstic acid which precipitates only the impure compound but not the pure vitamin [24 25] Mercuric chloride [6] gold chloride and $H_2PtCl_4$ also precipitate the vitamin from impure solutions The vitamin is however not precipitated by flavianic acid, rufianic acid Reinecke salts picric acid picrolonic acid barium salts [5] quinine or alkaloids [27] Alcohol insoluble salts are obtained from barium or calcium hydroxide

Vitamin H is adsorbed on charcoal (Norit and Carboraffin) but not on fuller s earth aluminum oxide acid clay or benzoic acid Vitamin H can be eluted with a mixture of pyridine methanol and water The methyl ester on the other hand can be purified by adsorption on aluminum oxide [27a]

Further purification of the vitamin can be accomplished by electro dialysis[28] and by high vacuum distillation [29]

By a combination of these procedures 1 1 mg crystallized biotin have been obtained from 250 kg of dried Chinese egg yolk [29]

## 5 Properties

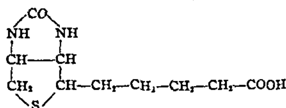
The free vitamin H is water and alcohol soluble but is relatively insoluble in chloroform ether and petroleum ether The vitamin is essentially heat stable, readily dialyzable and resistant to treatment with acid or alkali It is easily adsorbed on charcoal The isoelectric point appears to be between pH 3 and 3 5 The pure vitamin melts at 230–232° C and the methyl ester at 166–167° C Biotin is optically active $[\alpha]_D^{22} = +92°$

[20] J G Lease *Z Vitaminforsch* 5 110 (1936)
[21] J G Lease and H T Parsons *Proc Am Soc Biol Chem J Biol Chem* 105 I (1934)
[22] P György *Ibid* 119 XLIII (1937)
[23] J G Lease *Z Vitaminforsch* 5 110 (1936)
[24] P György R Kuhn and E Lederer *J Biol Chem* 131 745 (1939)
[25] C Drumel and L Hubert *Arch intern Physiol* 46 141 (1938)
[27] T W Birch and P György *J Biol Chem* 131 761 (1939)
[27a] V du Vigneaud K Hofmann D B Melville and P György *J Biol Chem* 140 643 (1941)
[28] P György D B Melville D Burk and V du Vigneaud *Science* 91 243 (1940)
[29] F Kögl and B Tönnis *Z physiol Chem* 242 43 (1936)

in 0 1 *N* NaOH It shows no characteristic absorption in the ultraviolet region [30]

## 6 Chemistry

The empirical formula of biotin is $C_{10}H_{16}O_3N_2S$ By means of electrodialysis it was shown that vitamin H is an ampholyte with acidic properties [31] The presence of a carboxyl group is indicated by the formation of esters such as the methyl ester Biotin contains a basic group since at pH = 1 the vitamin cannot be extracted with organic solvents [32] The nitrogens are present in urea configuration By treatment of biotin with barium hydroxide at 140° C a diamino carboxylic acid is obtained with the loss of one carbon atom and one oxygen atom By treatment of the diamino carboxylic acid with phosgene the urea group and thereby biotin is resynthesized The sulfur is present as a thio ether, since a sulfone results from peroxide oxidation Since biotin does not contain any double bonds it must contain a bicyclic ring system [32a] Upon oxidation of the diamino carboxylic acid with nitric acid or alkaline permanganate, adipic acid is isolated [32b] One of the carboxyl groups of the adipic acid is the carboxyl group originally present in biotin, since upon Curtius degradation of the biotin methyl ester followed by oxidation of the obtained amine no adipic acid could be isolated [32c] On the basis of these and additional evidences, [32d] biotin has been assigned the structure of 2 keto 3 4 imidazolido 2 tetrahydro thiophene *n* valeric acid (I) This conclusion has been confirmed by a total synthesis of biotin [33]




[30] V du Vigneaud K Hofmann D B Melville and J R Rachele *J Biol Chem* 140 763 (1941)

[31] T W Birch and P György *J Biol Chem* 131 761 (1939)

[32] H Kringstad and G Lunde *Ibid* 261 110 (1939)

[32a] F Kögl and L Pons *Z physiol Chem* 269 61 (1941) F Kögl and T J de Man *Ibid* 269 81 (1941) K Hofmann D B Melville and V du Vigneaud *J Biol Chem* 141 207 (1941) D B Melville K Hofmann and V du Vigneaud *Science* 94 308 (1941)

[32b] K Hofmann D B Melville and V du Vigneaud *J Am Chem Soc* 63 3237 (1941)

[32c] V du Vigneaud K Hofmann and D B Melville *J Am Chem Soc* 64 188 (1942)

[32d] Refer to review papers by V du Vigneaud *Science* 96 455 (1942) and K Hofmann *Advances in Enzymology* Vol III Interscience New York 1943 p 289

[33] S A Harris D E Wolf R Mozingo and K Folkers *Science* 97 447 (1943)

## 7 Industrial Methods of Preparation

Pure vitamin H is marketed in small quantities prepared by tedious isolation procedures Concentrates are also manufactured from liver, yeast and certain bacteria The preparation of the concentrates from liver is coordinated with the manufacture of the factor which prevents the development of pernicious anemia since the residues from this manufacture contain relatively high concentrations of vitamin H [33, 34, 35]

## 8 Specificity

Very little is known about the specificity of vitamin H The methyl ester is fully active Benzoylation and acetylation do not effect the activity probably because of the ability of the organism to hydrolyze these derivatives Vitamin H in its naturally occurring chemically bound form is also essentially active Inactive however is the biotin symplex with avidin (see page 476)

## 9 Determination

### (a) *Biological Methods*

There are only biological methods available for the determination of biotin The most valuable and accurate methods are those in which micro organisms are used and in which the biotin concentration is measured by the growth of yeast or of bacteria or by the amount of acid produced by specific organisms The historically important bio assay with rats is based upon a secondary deficiency disease brought about by egg white injury and is quite time consuming The chick can also be used for quantitative assays in a procedure which uses a primary biotin deficiency and which is more expedient than rat assays but less so than the micro biological methods

**Rat Tests** *Male albino rats are fed rations which are well balanced* except for a large proportion of egg white as the sole source of protein [36, 37] The average time necessary for the appearance of the first skin symptoms is five to seven weeks A rat growth method has also been used [38]

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[33] J G Lease and H T Parsons *Biochem J* 28 109 (1934)
[34] P György, *J Biol Chem* 131 733 (1939)
[35] J G Lease *Z Vitaminforsch* 5 110 (1936)
[36] M A Boas *Biochem J* 21 712 (1927)
[37] P György *J Biol Chem* 131 733 (1939)
[38] G Lunde and H Kringstad *J Nutrit* 19 [illegible] 1 (1940)

**Chick Test** The chick has been suggested as test animal for the determination of vitamin H since chicks can be depleted readily from this vitamin on a diet low in this constituent [39]

**Coenzyme R Assay** [40] In this test, the increase in respiration of rhizobium is measured upon addition of vitamin H to the culture medium This method is far more sensitive than the rat assay method for vitamin H

**Yeast Growth Method** The growth of special yeast strains such as *Saccharomycetes cerevisiae* is observed for example, by measuring the resulting turbidity in a photoelectric colorimeter[41] or nephelometer [42]

**Staphylococcus Growth Test** [43] The response of the addition of vitamin H preparations to a culture medium of *Staphylococcus aureus* has been suggested as a quantitative method for the assay of vitamin H

**Colostridum Butylicum Growth Test** [44] The organism *Colostridum butylicum* has been suggested for vitamin H assays The growth is measured by turbidity studies In this test only the free vitamin is measured and not the bound vitamin

**Acidimetric Lactobacillus Test** [45] The amount of lactic acid produced by *Lactobacillus arabinosus* cultivated in a medium containing all the other necessary growth factors can be used as a measure of the vitamin H content

## 10 Standards

One "Rat Unit" is the daily dose of a given preparation or foodstuff which in four weeks brings about complete cure of the egg white injury in rats (on a special diet) [46] This corresponds to an activity of 27 million Rat Units of vitamin H per gram of the methyl ester of biotin [47]

One "Saccharomycetes Unit"[48] (S U) is the amount of biotin which produces an increase of 100% in cell growth of a special strain of yeast under defined conditions One gram of biotin contains 25,000,000,000 Saccharomycetes Units

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[39] D M Hegsted J J Oleson R R Mills C A Elvehjem and E B Hart *J Nutrition* 20 599 (1940) S Ansbacher and M Landy *Proc Soc Exptl Biol Med* 48 3 (1941)

[40] F E Allison S R Hoover and D Burk *Science* 78 217 (1933) F E Allison and F W Minor *Soil Sci* 46 473 (1938)

[41] E E Snell R E Eakin and R J Williams *J Am Chem Soc* 62 175 (1940)

[42] F Kögl and B Tönnis *Z physiol Chem* 242 43 (1936)

[43] J R Porter and M J Pelczar *Science* 91 ο76 (1940)

[44] J O Lampen A A Kline and W H Peterson *Proc Am Soc Biol Chem* 1941 LXXIV

[45] F F Snell and L D Wright *Ibid* 1941 CXIX

[46] P György *J Biol Chem* 131 733 (1939)

[47] P György C S Rose K Hofmann D B Melville and V du Vigneaud *Science* 92 [illegible] (1940)

[48] F Kögl and B Tönnis *Z physiol Chem* 242 43 (1936)

## 11 Physiology of Plants and Microorganisms

Vitamin H is necessary for the growth of certain bacteria, for example, various strains of *clostridium*,[49, 50] *rhizobium*,[51, 52] *staphylococcus*[53, 54, 55] etc, and certain yeast strains[56] Other bacterial species have been shown to synthesize biotin in varying amounts[57] Vitamin H is also a true growth hormone for higher plants This fact is indicated by the occurrence of increased amounts in plant seeds and in the tips of roots and coleoptiles and has been proved experimentally with isolated pea roots[58] The roots of higher plants excrete biotin into the soil[59]

The role which biotin plays in plants and in microorganisms may be that of a true growth stimulant but an influence upon other factors is by no means excluded Thus, it has been observed that in yeast biotin increases fermentation more directly than respiration and respiration again more directly than growth[60] It has also been shown for *rhizobium* that the respiration can be markedly increased without concomitant growth by the addition of biotin to the culture medium[61]

## 12 Animal Physiology

### (a) *Metabolism of Vitamin H*

Vitamin H is easily absorbed from the intestinal tract The naturally occurring bound form seems to be equally well utilized perhaps due to hydrolysis in the intestine Some destruction appears to occur during the adsorption of biotin since the requirement by subcutaneous administration is only about one fifth of that required for oral administration

The animal and human organism is apparently able to store a certain amount of vitamin H especially in the liver and the kidneys On the other hand, newborn infants have practically no such reserves Similarly the secretion into milk although not negligible is markedly low Excretion in the feces has been observed but occurs predominantly through the urine

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[49] W H Peterson L E McDaniel and E McCoy *J Biol Chem* 133 LXXV (1940)
[50] E E Snell and R J Williams *J Am Chem Soc* 61 3594 (1939)
[51] P M West and P W Wilson *Enzymologia* 8 152 (1940)
[52] R Nilsson G Bjälfve and D Burström *Ann Landw Hochschule Schwedens* 7 301 (1939) *Naturwissenschaften* 26 661 (1938) 27 389 (1939)
[53] J R Porter and M J Pelczar *Science* 91 576 (1940)
[54] F Kögl and W J van Wagtendonk *Rec trav chim* 57 747 (1938)
[55] G A Hottle J O Lampen and A M Pappenheimer *J Biol Chem* 137 459 (1941)
[56] E E Snell R E Eakin and R J Williams *J Am Chem Soc* 62 175 (1940)
[57] M Landy and D M Dicken *Proc Soc Exptl Biol Med* 46 449 (1941)
[58] F Kögl and A J Haagen Smith *Z physiol Chem* 243 209 (1936)
[59] P M West *Nature* 144 1050 (1939)
[60] D Burk R. J Winzler and V du Vigneaud *Proc Am Soc Biol Chem* 1941 XXI
[61] F E Allison S R Hoover and D Burk *Science* 78 217 (1933)

During vitamin H deficiency the tissues, for example, of chicks are practically devoid of this vitamin[62]

### (b) *Physiological Action of Vitamin H*

The physiological action of vitamin H is not clearly understood A close relationship between this vitamin and fat metabolism has been postulated A certain increase in the fat and cholesterol synthesis in the liver of rats upon feeding vitamin H has been observed This abnormal activity can be prevented by the addition of lipocaic (an internal secretion of the pancreas) to the diet[63]

Biotin is made unavailable to the living organism (experiments with rats, chicks and yeast) by the formation of a complex with a protein constituent of raw egg white, and the typical syndromes of a biotin deficiency occur when, instead of the free biotin, the protein symplex is administered (This is the historically important egg white toxicity) The combination of biotin with the protein is stoichiometric and biotin cannot be recovered by dialysis The protein which has the peculiar capacity of binding biotin is called 'avidin and has been obtained in crystalline form[64]

## 13 Avitaminosis

Rats develop peculiar and impressive skin changes on a diet deficient in vitamin H These are accompanied by progressive emaciation and finally led to death The skin lesions are differentiated from the pellagra or acrodynia syndrome since they are not predominantly a peripheral dermatosis[6] The dermatitis can best be regarded as a seborrheid desquamative type At the same time, biotin is involved to a certain extent in the maintenance of the normal pigment metabolism of the fur in rats and mice[66] In human infants[67] and in rats[68] a deep brown pigmentation has been observed on the back Male rats exhibit a greater sensitivity to vitamin H deficiency than female rats[69, 65] A high incidence of a special type of pneumonia which could be improved and even completely cured by the

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[62] R E Eakin W A McKinley and R J Williams *Science* 92 224 (1940)
[63] E W McHenry and G Gavin *Proc Am Soc Biol Chem* 1941 LXXXVII
[64] R E Eakin E E Snell and R J Williams *J Biol Chem* 136 801 (1940) 140 535 (1941)
D Pennington E E Snell and R E Eakin *J Am Chem Soc* 64 469 (1942)
[65] P György *J Biol Chem* 131 733 (1939)
[66] P Gyorgy and C E Poling *Proc Soc Exptl Biol Med* 45 773 (1940)
[67] E Moro *Ekzema Infantum und Dermatitis Seboerhoides* Berlin 193- p
[68] H T Parsons *J Biol Chem* 90 351 (1931)
[69] M A Boas *Biochem J* 21 712 (1927)

administration of vitamin H[65] has been noted[70] Certain disturbances of the nervous system in rats on a vitamin H deficient diet have been observed[71] Furthermore biotin deficiency in rats causes a typical denudation around the eyes This syndrome is known as the 'spectacle eye condition'[71a] and is prevented and cured by the administration of biotin[71b]

Vitamin H deficiency in chicks results in a specific dermatitis[71c] The bottoms of the feet become rough and calloused and in more severe cases encrusted and hemorrhagic cracks appear In addition, mandibular lesions occur in the corners of the mouth and around the beak and the eyelids become swollen and stick together

Experimental biotin deficiency in man on a diet in which about 30% of the total calories was supplied by desiccated egg white, resulted in the occurrence of a number of clinical symptoms similar to those of spontaneous avitaminosis A striking ashy pallor of the skin and mucous membranes appeared followed by an increasing dryness of the skin with marked reticulation and a fine branny desquamation Extreme lassitude and somnolence muscle pains precordial distress and anorexia were observed Parenteral administration of biotin cured these symptoms rapidly[71d]

In man vitamin H therapy has given curative results in a few isolated cases of acne vulgaris[72] and rosacea and of furunculosis Certain cases of baldness in men are caused by seborrhoic conditions and can be improved by vitamin H administration More severe cases of seborrhea (overaction of the sebaceous glands) seem to be related to the skin disease called psoriasis which consists of an eruption of circumscribed rounded patches occurring chiefly on the elbows and knees scalp and back Encouraging results have been attained in these cases by administration of vitamin H

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[70] M Gundel P György and W P g l Z Hyg Infektionsk ankh 113 629 (1932)

[71] C M Findlay and R O Stern Arch Disease Childhood 4 1 (1929) J G Lea e H T ... rson and F Kelly Biochem J 31 433 (1937)

[71a] J Goldberger and R D Lill Pub Health Rep U S P H S 41 102 (1926) A Bourquin and H C Sherman J Am Chem Soc 53 3501 (1931) H E Robinson and R C Newton Abstracts Div of Biol Chem A C S Kansas City April 13 17 1936 S Lepkovsky T H Jukes and M E Krause J Biol Chem 115 557 (1936) B Sjollema Acta Brevia Neerland Physiol Pharmacol Microbiol 102 148 (1937) Tijdschr Diergeneesk 64 986 (1937) P Karrer ... Laszt and F Verzár Arch ges Physiol 239 644 (1937) E M Mackay and R H Barnes Proc Soc Exptl Biol Med 46 353 (1941) J J Oleson H R Bird C A Elvehjem and E B Hart J Biol Chem 127 23 (1939) J J Oleson C A Elvehjem and E B Hart Proc Soc Exptl Biol Med 43 161 (1940) P F Pavcek and H M Baum Science 93 50 (1941)

[71b] E Nielsen and C A Elvehjem Proc Soc Exptl Biol Med 48 349 (1941)

[71c] D M Hegsted J J Oleson R C Mills C A Elvehjem and E B Hart J Nutrition 20 599 (1940) D M Hegsted R C Mills G M Briggs C A Elvehjem and E B Hart Ibid 23 175 (1942)

[71d] V P Sydenstricker S A Singal A P Briggs N M DeVaughn and H Isbell Science 95 176 (1942)

[72] W M ... J Invest Dermatol 2 205 (1939)

## 14 Requirements

Vitamin H is required by all animals investigated, such as rats, chicks[73] guinea pigs, rabbits, monkeys[74] dogs[75] and man Man needs 50 Rat Units of vitamin H when administered subcutaneously or about three to five times this amount when given *per os*[76] Cattle do not necessarily need an external supply of vitamin H since the microorganisms in the rumen synthesize the vitamin[77] The requirements of microorganisms (bacteria) have been discussed previously (see page 475)

Men have responded favorably to parenteral administration of 150–300 gamma of biotin per day Chicks need about 2 5–10 gamma and rats about 1–3 gamma per day

[73] A T Ringrose L C Norris and G F Heuser *Poultry Sci* 10 166 (1931)

[74] J G Lease H T Parsons and E Kelly *Biochem J* 31 433 (1937)

[75] P György *J Biol Chem* 131 733 (1939)

[76] *P Gyorgy Ibid 119 XLIII (1937) F Schultz Med u Chem Abhandl med chem Forschung stätten I G Farbenind* 3 143 (1936) P György and C S Rose *Proc Soc Exptl Biol Med* 43 73 (1940)

[77] M I Wegner A N Booth C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 45 769 (1940)

# THE GROUP OF
# VITAMINS K

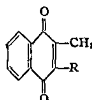
# THE GROUP OF VITAMINS K[1]

## 1 Nomenclature and Survey

**Names**

Vitamins K[2]
Phylloquinones[3] $\alpha$, $\beta$, etc.
Koagulations Vitamin (German and Scandinavian term from which the present term "vitamin K" was derived.)
Coagulation vitamin
*Prothrombin factor*
Antihemorrhagic vitamin

**Structure**



Vitamin $K_1$ $\quad R = -CH_2\,CH{=}\overset{CH_3}{\overset{|}{C}}\,CH_2\,[CH_2\,CH_2\,\overset{CH_3}{\overset{|}{C}H}\,CH_2]\;CH_2\,CH_2\,\overset{CH_3}{\overset{|}{C}H}\,CH_3$

Vitamin $K_2$ $\quad R = -CH_2\,[CH{=}\overset{CH_3}{\overset{|}{C}}\,CH_2\,CH_2]_5\,CH{=}\overset{CH_3}{\overset{|}{C}}\,CH_3$

2 Methyl 1 4 naphthoquinone $\quad R{=}H$

## 2 Chronology

1929 DAM[4] observed subcutaneous and intramuscular hemorrhages in chicks raised on an artificial diet

1934 DAM and SCHØNHEYDER[5] concluded that the hemorrhagic conditions of the chicks were caused by an avitaminosis and called the new factor vitamin K[6]

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[1] H R Butt and A M Snell, *Vitamin K*, Philadelphia, 1941
[2] H Dam, *Nature* 135 652 (1935)
[3] P Karrer and A Geiger, *Helv Chim Acta* 22 945 (1939)
[4] H Dam, *Biochem Z* 215 475 (1929), 220 158 (1930)
[5] H Dam and F Schønheyder, *Biochem J* 28 1355 (1934)
[6] H Dam, *Nature* 135 652 (1935)

1935 DAM SCHØNHEYDER and co worker[7] found that vitamin K takes part in restoring and maintaining the normal prothrombin level of blood

1938 WARNER BRINKHOUS and SMITH[8] BUTT SNELL and OSTERBERG[9] and DAM and GLAVIND[10] demonstrated clinically the usefulness of vitamin K for man

1939 DAM KARRER and co-workers[11] isolated pure vitamin $K_1$ and DOISY and co-workers[12] isolated pure vitamin $K_2$ The synthesis of vitamin $K_1$ was achieved independently in three different laboratories namely in those of ALMQUIST[13] DOISY[14] and FIESER[15] ANSBACHER and FERNHOLZ[16] discovered the anti hemorrhagic activity of 2 methyl 1 4 naphthoquinone

1940 DOISY and co workers[17] elucidated the chemical structure of vitamin K

## 3 The Group of Vitamins K

The existence of only two naturally occurring vitamins K of high activity namely vitamins $K_1$ and $K_2$, has been proved with certainty Phthiocol a naturally occurring compound, is also somewhat active but in considerably higher amounts The inclusion of this compound into the class of naturally occurring vitamins K depends upon the proof that this compound is not an artefact from the alkaline hydrolysis procedure used for its isolation[18, 19] It is, however, conceivable that besides vitamins $K_1$ and $K_2$ other members of similar structure occur naturally, especially of the type with $\beta$ unsaturated isoprenoid side chains in the 3 position of 2 methyl 1,4 naphthoquinone, such as 3 farnesyl or 3 geranyl derivatives[20]

Indication for the occurrence of at least one other factor has been obtained through the isolation of colorless fractions[21, 22] This factor is highly active and resembles in its behavior the oxides of vitamins K It is, therefore, possible that this compound or compounds are oxides obtained during the procedure of isolation

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7 H Dam *Nature* 135 652 (1935) F Schønheyder *Ibid* 135 652 (1935) H Dam F Schønheyder and F Tage Hansen *Biochem J* 30 1075 (1936)

8 E D Warner K M Brinkhous and H P Smith *Proc Soc Exptl Biol Med* 37 628 (1938)

9 H R Butt A M Snell and A E Osterberg *Proc Staff Meetings Mayo Clinic* 13 74 (1938)

10 H Dam and J Glavind *Lancet* 234 720 (1938) *Acta Med Scand* 96 108 (1938)

11 H Dam A Geiger J Glavind P Karrer W Karrer F Rothschild and H Salomon *Helv Chim Acta* 22 310 (1939)

12 R W McKee S B Binkley D W MacCorquodale S A Thayer and E A Doisy *J Am Chem Soc* 61 1295 (1939)

13 H J Almquist and A A Klose *Ibid* 61 2557 (1939)

14 S B Binkley L C Cheney W F Holcomb R W McKee S A Thayer D W MacCorquodale and E A Doisy *Ibid* 61 2558 (1939)

15 L F Fieser *Ibid* 61 2559 (1939)

16 S Ansbacher and E Fernholz *Ibid* 61 1924 (1939)

17 S B Binkley R W McKee S A Thayer and E A Doisy *J Biol Chem* 133 721 (1940)

18 L F Fieser *J Am Chem Soc* 61 2559 (1939)

19 L F Fieser *Ibid* 61 3467 (1939)

20 L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 659 (1941)

21 S Ansbacher E Fernholz and H B MacPhillamy *Proc Soc Exptl Biol Med* 42 655 (1939) E Fernholz S Ansbacher and M L Moore *J Am Chem Soc* 61 1613 (1939)

## 4 Occurrence

Generally speaking vitamins K occur only in plants and in micro organisms Which one of the group of vitamins K is present in each source is largely unknown but it is assumed that the green leaves of plants contain predominantly or solely vitamin $K_1$ whereas the microorganisms contain vitamin $K_2$

The best sources of vitamin $K_1$ are the green leafy tissues of alfalfa spinach cabbage,[23, 24] kale cauliflower nettle chestnut[5] etc Vitamin $K_1$ is also present in tomatoes hempseed seaweed[5] and soybean oil[26] Fruits and cereals are very poor sources of the vitamin

Vitamin $K_2$ occurs in many microorganisms especially in most bacteria[27] while molds yeasts and fungi contain no, or practically no vitamin K From a practical standpoint it is important that the microorganisms in the intestinal tract contain abundant quantities Most putrefied animal and plant materials contain high amounts due to bacterial growth

Animal materials contain very little vitamin K although milk and eggs contain small amounts Hog liver[28] is the richest animal source found so far The livers of chicks[28, 29, 30] and of rats[28] contain very little vitamin K

## 5 Isolation

The isolation of vitamin $K_1$ from for example alfalfa leaf meal is carried out by first extracting the dried plant material with petroleum ether, hexane[31] or acetone[32] followed by an adsorption of the chlorophyll present on zinc carbonate[33] magnesium oxide[34] or phosphotungstic acid[34] Upon cooling the remaining solution some material precipitates and is discarded The solution is then subjected to fractional distillation[35, 36] Vitamin $K_1$ distills between 120° and 140° C at $10^{-4}$ mm Hg Several compounds especially sterols are separated from the distillate by crystallization from

[23] H Dam Nature 135 65 (193 ) Bioche J 29 1 74 (1935)
[24] H J Almquist and E L R Stokstad Nature 136 31 (193 ) J B l Che 111 10 (193 )
[25] H Dam and J Glavind Biochem J 32 48 (1938)
[26] H J Almquist and E L R Stokstad J Nutrition 14 235 (1937)
[27] H J Almquist C F Pentler and E Mecchi Proc Soc Exptl Biol Med 38 336 (1938)
[28] H Dam and F Schønheyder Biochem J 30 897 (1936)
[29] H Dam and J Glavind Lancet 234 720 (1938)
[30] H J Almquist and E L R Stokstad J Nutrition 12 329 (1936)
[31] H J Almquist and E L R Stokstad Ibid 12 329 (1936)
[32] H Dam and F Schønheyder Biochem J 30 897 (1936)
[33] P Karrer A Geiger R Legler A Rüegger and H Salomon Helv Chim Acta 22 1464 (1939)
[34] A A Klose and H J Almquist J Am Chem Soc 61 53 (1939)
[35] B Riegel C I Schweitzer and P C Smith J Biol Chem 129 495 (1939)
[36] H J Almquist Ibid 120 63 (1937) H Dam and L Lewis Biochem J 31 17 (193 )

acetone By application of the chromatographic adsorption technic using first dehydrated magnesium sulfate and then zinc carbonate the pure vitamin can be obtained [33] Other adsorbing agents such as calcium sulfate,[37] weakly acidic fuller's earth (Florex) [38] Permutit,[39, 40] Darco [39] etc have been used for the isolation of both vitamins $K_1$ and $K_2$

The isolation of vitamin K according to these procedures is relatively difficult A very simple method has however been devised for the isolation of vitamin K in the reduced form [41] This consists in reducing a crude vitamin K concentrate in alcohol solution with sodium hydrosulfite and extracting the reduced vitamin with petroleum ether The solvent solution is then extracted with 5% sodium hydroxide containing hydrosulfite which removes considerable amounts of foreign matter The petroleum ether solution is then extracted with Claisen s alkali solution [42] prepared from potassium hydroxide, water and methanol, to which hydrosulfite has been added The alkaline solution is diluted with water and the reduced vitamin is extracted with ether The ether solution is washed with hydrosulfite solution and the reduced vitamin is obtained by evaporation of the solvent The vitamin itself is obtained from the reduced compound by shaking in ether solution with silver oxide and magnesium sulfate

Vitamin K can also be isolated in the form of the diacetate of the reduced form which is prepared by dissolving the vitamin in acetic anhydride and adding zinc dust and pyridine [43, 44]

## 6 Properties

The vitamins K are soluble in most of the common organic solvents, especially in ether, petroleum ether, hexane acetone, etc but are insoluble in water and only sparingly soluble in methanol The vitamins are essentially thermostable [45] They are very sensitive to alkali[46] and to light from various sources, such as sunlight,[47, 48] the illumination from ordinary

[37] H Dam *Z Vitaminforsch* 8 248 (1938 39)
[38] B Riegel C E Schweitzer and P C Smith *J Biol Chem* 129 495 (1939)
[39] S B Binkley D W MacCorquodale S A Thayer and E A Doisy *Ibid* 130 219 (1939)
[40] R W McKee S B Binkley S A Thayer D W MacCorquodale and E A Doisy *Ibid* 131 327 (1939)
[41] L F Fieser *J Am Chem Soc* 61 3467 (1939)
[42] L Claisen *Ann* 418 96 (1919)
[43] L F Fieser *J Am Chem Soc* 61 3467 (1939)
[44] S B Binkley D W MacCorquodale L C Cheney S A Thayer R W McKee and E A Doisy *Ibid* 61 1612 (1939)
[45] H J Almquist *J Biol Chem* 120 635 (1937)
[46] H J Almquist and E L R Stokstad *J Nutrition* 14 235 (1937)
[47] D W MacCorquodale S B Binkley R W McKee S A Thayer and E A Doisy *Proc Soc Exptl Biol Med* 40 482 (1939)
[48] P Karrer and A Geiger *Helv Chim Acta* 22 945 (1939)

Mazda light bulbs[47] and ultraviolet light[49] The vitamins K exhibit typical absorption characteristics in the ultraviolet region with maxima at 243 248 261 270 and 328 mμ (Fig 24)

Vitamin $K_1$ is a yellow oil which melts at about −20° C The redox potential is $E_m = +0.005$ volt Vitamin $K_1$ exhibits a white fluorescence in the light of an argon lamp[50] This phenomenon has not been observed in simple naphthoquinones without side chains in the 3 position

Vitamin $K_2$ is a yellow crystalline solid m p 53 5–54 5° C[51]

**2-Methyl-1,4-naphthoquinone** is a lemon yellow crystalline powder with faint but characteristic odor melting at 106° C It is soluble in

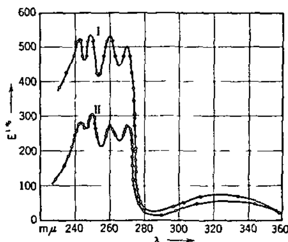



Fig 24 —Absorption spectra of vitamin $K_1$ (I) and of vitamin $K_2$ (II) (D T Ewing J M Vandenbelt and O Kamm)

water to the extent of 0 13 mg per ml The absorption spectrum of 1 4-naphthoquinone and the diacetate of the hydroquinone are shown in Fig 25

## 7 Chemical Constitution

### (a) *The Constitution of Vitamin $K_1$*

Vitamin $K_1$ has the empirical formula $C_{31}H_{46}O_2$ and, according to potentiometric titrations with sodium hydrosulfite a molecular weight of 450[52] The presence of the oxygen atoms as quinone oxygens was deduced from the redox potential which proved to be very similar to that

[49] H J Almquist *J Biol Chem* 117 517 (1937)
[50] H J Almquist and A A Klose *J Am Chem Soc* 61 7 (1939)
[51] S B Binkley R W McKee S A Thayer and E A Doisy *Ibid* 133 7 1 (1940)
[52] P Karrer and A Geiger *Helv Chim Acta* 22 94 (1939)

of many 1,4 quinones such as, for example, the anthraquinone hydroanthraquinone system[50] The presence of a quinoid structure was also suspected from the absorption spectrum of the vitamin[53] which proved to be very similar to 2,3 disubstituted 1,4 naphthoquinones Further indications were the instability of the vitamin toward alkali and light and finally the absorption of eight atoms of hydrogen upon catalytic hydrogenation[3] (I → II) The reduced compound is colorless, but upon exposure to light it is oxidized to a yellow compound (III) the color of which is similar to that of the vitamin itself This suggests that the original

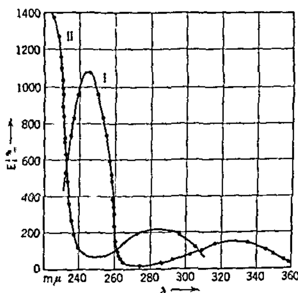
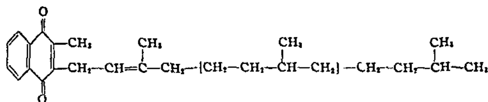



Fig 25 —Absorption spectra of 1 4-naphthoquinone (I) and the diacetate of 1 4 naphthohydroquinone (II) (D T Ewing J M Vandenbelt and O Kamm)

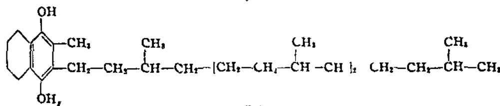
vitamin is a quinone which is reduced catalytically and partially reoxidized Further proof was furnished by reductive acetylation which yielded a crystalline diacetate (IV) (formula on page 488) from which the vitamin could be recovered by hydrolysis by means of a Grignard reaction, followed by air oxidation[54] The color of the quinone is yellow, which suggests a 1 4 position of the oxygen atoms, since the only other possible quinones namely the 1 2 quinones, are red

[53] R W McKee S B Binkley D W MacCorquodale S A Thayer and E A Doisy *J Am Chem Soc* 61 1295 (1939)
[54] S B Binkley D W MacCorquodale L C Cheney S A Thayer R W McKee and E A Doisy *Ibid* 61 1021 (1939)

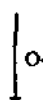


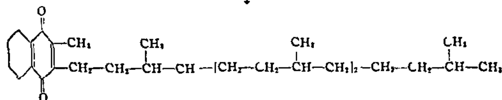
(I)





(II)





(III)

Vitamin $K_1$ upon oxidation with an excess of chromic acid yielded phthalic acid (V)[55] This shows that the aromatic non quinoid ring has no side chains attached and suggests that a 1 4 naphthoquinone is present which then must be substituted in the 2 and 3 positions Mild chromic acid oxidation yielded 2 methyl 1 4 naphthoquinone 3 acetic acid (VI)[55] which was identified by comparison with a synthetic specimen[56] Similarly chromic acid oxidation of the diacetate of dihydro vitamin $K_1$ yielded 1 4 diacetoxy 2 methyl naphthalene 3 acetic acid,[56] the methyl ester of which proved to be identical with the synthetic compound of the indicated structure

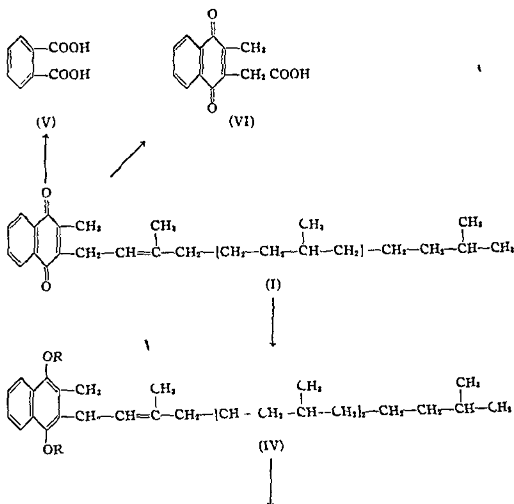
As indicated above catalytic hydrogenation of vitamin $K_1$ yielded a hydroquinone by absorption of four mols of hydrogen Since hydrogena

[55] D W MacCorquodale S B Binkley S A Thayer and E A Doisy *J Am Chem Soc* 61 19 8 (1939)

[56] S B Binkley L C Cheney W F Holcomb R W McKee S A Thayer D W MacCorquodale and E A Doisy *Ibid* 61 2558 (1939)

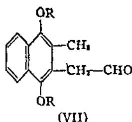
tion of a naphthoquinone nucleus would require only three mols, the presence of a double bond in a side chain was indicated This was proved by oxidation of the diacetate of dihydro vitamin $K_1$ with ozone which yielded a ketone, $C_{18}H_{36}O$ (VIII) which was found to be identical[56] with the corresponding ketone obtained by oxidation of phytol[57] The constitution of this ketone is 2,6,10 trimethyl pentadecanone 14 The other reaction product from the ozonolysis is 1,4 diacetoxy 2 methyl naphthalene 3 acetaldehyde (VII) which was characterized as the semicarbazone[58, 59]

These degradation reactions prove conclusively the structure of vitamin $K_1$ as 2 methyl 3 phytyl 1,4 naphthoquinone

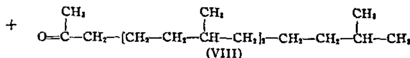



(Formula continued on following page)

[57] F G Fischer and K Löwenberg *Ann* 464 69 (1929)
[58] S B Binkley R W McKee S A Thayer and E A Doisy *Proc Am Soc Biol Chem* 1940 XII
[59] S B Binkley R W McKee S A Thayer and E A Doisy *J Biol Chem* 133, 721 (1940)




(VII)




(VIII)

### (b) The Constitution of Vitamin $K_2$

The methods used for the elucidation of the structure of vitamin $K_1$ and the knowledge gained from the proof of the structure of this vitamin were successfully applied for the determination of vitamin $K_2$

Vitamin $K_2$ has the empirical formula $C_{41}H_{56}O_2$ Upon reductive acetylation a diacetate of dihydro vitamin $K_2$ is formed[60] (I → II) which proves the presence of the two oxygens in a quinone structure The quinone structure is also revealed by the absorption spectrum, which is very similar to that of vitamin $K_1$ (See Fig 24 on page 485)

Upon catalytic hydrogenation vitamin $K_2$ absorbs 9 mols of hydrogen[61] Since three mols are required for reduction of the quinone structure the presence of six double bonds in the side chains is indicated This assumption is confirmed by the fact that dihydro vitamin $K_2$ diacetate adds 12 atoms of bromine These six double bonds are not in conjugation to each other since no addition product is formed with maleic anhydride and since the ultraviolet absorption spectrum does not indicate the presence of any conjugated double bonds in addition to the quinone structure

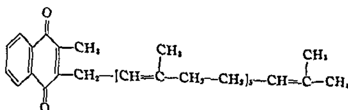
Oxidation of vitamin $K_2$ with ozone[6, 62] yields 1 4 diacetoxy 2 methyl acetaldehyde (III) which was characterized as the semicarbazone Levulin aldehyde (IV) was further isolated from the oxidation product and identified as the bis 2 4 dinitro phenyl hydrazone Assuming that 1 mol of vitamin $K_2$ would yield 5 mols of levulin aldehyde this compound was obtained in an 81% yield Acetone (V) was obtained also from the ozonolysis in the form of its 2 4 dinitro phenyl hydrazone in a yield of 56% assuming that one mol of acetone originates from one mol of the vitamin

[60] S B Binkley D W MacCorquodale L C Cheney S A Thayer R W McKee and E A Doisy *J Am Chem Soc* 61 1612 (1939)

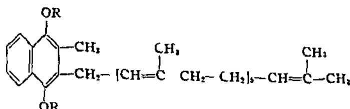
[6] R W McKee S B Binkley D W MacCorquodale S A Thayer and E A Doisy *Ibid* 61 1295 (1939)

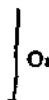
[61] S B Binkley R W McKee S A Thayer and E A Doisy *Proc Am Soc Biol Chem* 1940 XII

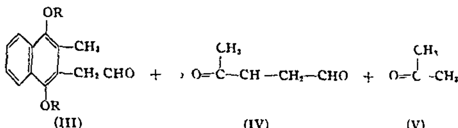
[62] S B Binkley R W McKee S A Thayer and E A Doisy *J Biol Chem* 133 721 (1940)









On the basis of these degradation products vitamin $K_2$ has the constitution (I) of a 2 methyl 3 difarnesyl naphthoquinone 1,4[64]

## 8 Synthesis

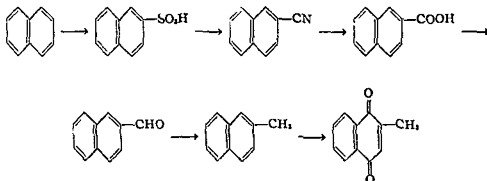
### (a) *Synthesis of 2-Methyl 1,4 naphthoquinone*

The simplest method for the synthesis of 2 methyl 1,4 naphthoquinone is the oxidation of 2 methyl naphthalene The latter compound is a by product obtained from the coal tar industry[65] The oxidation is best accomplished by means of chromic acid in acetic acid[66, 67] at temperatures

[63] S B Binkley R W McKee S A Thayer and E A Doisy *J Biol Chem* 133 721 (1940)
[64] P Karrer and A Epprecht *Helv Chim Acta* 23 272 (1940)
[65] E A Coulson *J Soc Chem Ind* 60 123 (1941)
[66] K Fries and W Lohmann *Ber* 54 2918 (1921)
[67] R J Anderson and M S Newman *J Biol Chem* 103 406 (1933)

below 50° C[68, 69] This oxidation can however also be carried out by other oxidizing agents such as molecular oxygen (air), hydrogen peroxide[70] etc Yields between 30% and 60% of theory have been reported for this oxidation

2 Methyl 1,4 naphthoquinone has also been synthesized by a simple and practical reaction sequence from naphthalene[71] Naphthalene is sulfonated in the β position and the sodium salt yields β naphthoic acid nitrile upon treatment with $K_4Fe(CN)_6$ The nitrile is hydrolyzed to the free β naphthoic acid the Ba salt of which gives upon distillation with barium formate the corresponding aldehyde in 60% yield Clemmensen reduction of the oxo compound yields 2 methyl naphthalene which is converted by oxidation into 2 methyl naphthoquinone




A total synthesis of 2 methyl 1 4 naphthoquinone has been accomplished[72] by condensing benzene by means of aluminum chloride with the anhydride of methyl succinic acid which is obtained from either citric acid or *d* tartaric acid The reaction product α methyl β benzoyl propionic acid, is reduced by the Clemmensen method to yield α methyl γ phenyl butyric acid Ring closure of its chloride with $AlCl_3$ yields 2 methyl α tetralone which is converted into 2 methyl 1 2,3 4-tetrahydro naphthalene by reduction Dehydrogenation with sulfur or with selenium yields 2 methyl naphthalene which is oxidized to the quinone

[68] L I Smith and I M Webster *J Am Chem Soc* 59 60 (1937)

[69] L F Fieser W P Campbell E M Prey and M D G tes *Ib d* 61 2559 3216 (1939)

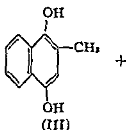
[70] R T Arnold and R Larson *J O g Chem* 5 250 (1940)

[71] P P T Sah W B üll and H Hol en *Ber* 73 762 (1940) I I T Sah *Rec t av chim* 59 161 (1940)

[72] P P T Sah and W Brüll *Be* 73 1430 (1940)

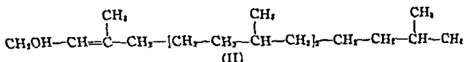
### (*b*) *Synthesis of Vitamin* $K_1$

The synthesis of vitamin $K_1$ (I) has been carried out from 2 methyl 1,4 naphthoquinone and phytol (II) by a number of different methods. Thus the synthesis has been effected from these compounds alone,[73] or, better, from 2 methyl 1,4 naphthohydroquinone (III) and phytol in the



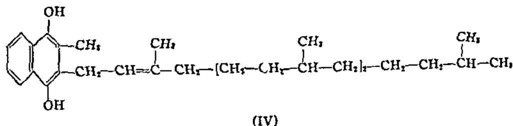
(III)

+



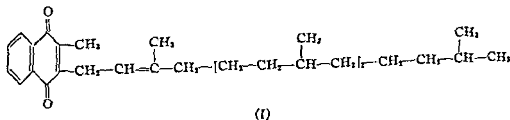
(II)





(IV)





(I)

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[73] H J Almquist and A A Klose J Am Chem Soc 61 2557 (1939)

presence of catalysts, such as oxalic acid or trichloro acetic acid[74] using dioxane as a solvent Another variation is to use the mono sodium salt of 2 methyl 1,4 hydroquinone and phytyl bromide[75] The hydroquinone has also been condensed with phytol in benzene solution using zinc chloride as the condensing agent Vitamin $K_1$ hydroquinone (IV) is obtained from the methods involving the use of the hydroquinone and is then oxidized to the vitamin (I) by air or silver oxide

The best yields can be obtained by working under mildly acidic conditions In the presence of strong acids such as mineral acids, the condensation is carried further giving rise to compounds of the vitamin E type Condensation *in alkaline medium, on the other hand, gives low* yield and undesirable dark brown reaction mixtures from which it is difficult to isolate the pure vitamin $K_1$

Vitamin $K_1$ has also been synthesized in low yields from phthiocol in its reduced form, and phytol[76]

### (c) *Synthesis of Vitamin $K_2$*

The synthesis of vitamin $K_2$ has not been accomplished as yet

## 9 Industrial Methods of Preparation

The concentrates of natural vitamins K such as alfalfa concentrates are still of considerable industrial interest, since they contain the natural vitamin $K_1$ which is clinically used Synthetic vitamin $K_1$ is available, prepared according to the methods previously described but is not widely used due to the high cost The synthetic 2 methyl naphthoquinone, which is at least as active although at high doses somewhat more toxic than the natural vitamins K is offered on the market and is used clinically

Water soluble forms of vitamin K have been introduced on the market While the natural vitamins and 2 methyl naphthoquinone are fat soluble and require the presence of bile acids for proper absorption from the intestinal tract the water soluble forms are absorbed as such and are also important for intravenous administration The following water soluble compounds of vitamin K action are used 2 methyl 1,4 naphtho

[74] L F Fieser *J Am Chem Soc* 61 2559 (1939)

[75] ? B B kley l C Ch ney W F Holcomb R W McK S A Th yer D W MacCorquodale nd F A Do sy *Ibid* 61 2558 (1939) D W M eCorq od l L C Cheney S B B nkl y W F Holcomb R W McKee S A Thayer and E A Do sy *J Biol Chem* 131 357 (1939)

[76] M T hl r L F Fieser and N I W ndler *J Am Ch m Soc* 62 198? (1940)

hydroquinone 3 sodium sulfonate,[77] 4 amino 2 methyl 1 naphthol hydrochloride[78, 79, 80] and sodium 2 methyl 1,4 naphthohydroquinone diphosphate[81, 82, 83]

## 10 Biogenesis

The vitamins K are synthesized by plants and by certain microorganisms In plants, the site of synthesis is in the green leaves Thus the tops of carrots contain a considerable amount of vitamin K while the roots contain practically none[84] The synthesis is greatly influenced by sunlight Peas grown in the dark contain only small amounts while control plants raised in light contain considerably more[85] The outer leaves of cabbage contain about four times more vitamin K than the inner leaves[85]

The synthesis of vitamin $K_1$ is presumably accomplished by methods similar to those used in the laboratory, that is, using phytol and methyl naphthoquinone as building units[86] Vitamin $K_2$ is synthesized in certain microorganisms which live on vitamin K free diets[87] Coli bacteria synthesize vitamin K on a synthetic medium which contains as organic constituents only glucose citrate and asparagin[88]

## 11 Specificity

The physiological activity of the vitamins $K_1$ and K is by no means restricted to these compounds A number of other similar compounds have the same property From the vast amount of work done to elucidate the specificity of the vitamin K action the following conclusions can be drawn The quinones may be converted into the corresponding hydroquinones, their esters and ethers without essential loss of efficacy as long as the derivatives can be reconverted into the quinones under more or less simple conditions which also may occur in the organism The unsub

[77] M B Moore and I J Kirchmeyer quoted in H R Butt and A M Snell *Vitamin K* Philadelphia 1941 M B Moore *J Am Chem Soc* 63 2049 (1941)

[78] E A Doisy D W MacCorquodale S A Thayer S B Binkley and R W McKee *Science* 90 407 (1939)

[79] H J Almquist and A A Klose *Proc Soc Exptl Biol Med* 45 55 (1940)

[80] D Richtert S A Thayer R W McKee S B Binkley and E A Doisy *Ibid* 44 601 (1940)

[81] L F Fieser and E M Frey *J Am Chem Soc* 62 228 (1940)

[82] R H K Foster J Lee and U V Solmssen *Ibid* 62 453 (1940)

[83] S Ansbacher E Fernholz and M A Dolliver *Proc Soc Exptl Biol Med* 43 65 (1940)

[84] H J Almquist *J Biol Chem* 117 517 (1937)

[85] H Dam and J Glavind *Biochem J* 32 485 (1938)

[86] L F Fieser W P Campbell and E M Frey *J Am Chem Soc* 61 2206 (1939) H J Almquist C F Pentler and E Mecchi *Proc Soc Exptl Biol Med* 38 336 (1938) A I Osterberg *Proc Staff Meetings Mayo Clinic* 13 72 (1938)

[88] H Dam J Glavind S Orla Jensen and A D Orla Jensen *Naturwissenschaften* 29 287 (1941)

stituted benzene ring of the vitamin molecule cannot be substituted without loss of activity The 2 methyl group cannot be replaced by hydrogen or higher alkyl groups without considerable decrease in activity The sub stituents on the 3 position, however can vary considerably and the change in efficacy is only slight The most remarkable fact is that when the substituent in 3 position is hydrogen, that is when 2 methyl 1 4 naphtho quinone is tested an activity of from two to four times that of vitamin $K_1$ per weight unit is observed This unique activity of 2 methyl naphtho quinone has caused widespread interest which led to the introduction of this compound into clinical therapy Its unusual efficacy has been ex plained by the hypothesis that this compound itself does not act as a vitamin in the organism but that it is converted in the organism into a quinone of the true vitamin K type On the other hand it has been postulated that the vitamins $K_1$ and $K_2$ owe their activity to their degrada tion in the organism to 2 methyl 1 4 naphthoquinone[89] While the latter hypothesis appears less attractive no experimental proof for the validity of the former can be offered other than the fact that such a synthesis is easily accomplished in the laboratory and that the building units for a side chain of the vitamin $K_1$ or $K_2$ type are readily available

The effect of variations of the side chain in 3 position are noteworthy A double bond in the $\beta$ $\gamma$ position contributes to the potency while un saturation at points more remote from the quinoid nucleus is without influence A branched side chain built from isoprene units is more active than a strictly straight chain and maximum activity is reached when some 20 or 30 carbon atoms are present

Finally the fact that the activity of the 2 3-oxides of the vitamins K is almost equivalent to that of the vitamins is noteworthy Compounds which may be identical with these have been isolated from natural ma terials but their identity has not been established as yet and the possi bility of an artefact during the process of isolation cannot be ruled out

These conclusions have been drawn from a large number of experiments with many compounds In the following tables comparative data are given for the minimum effective dose of various series of compounds which are chemically closely related The effective dose in these tables is defined as the minimum amount of material which, when administered in 0 1 cc of peanut oil, will reduce the blood clotting times of 60 to 80% of vitamin K-deficient chicks to less than 10 minutes during an 18 hour period[90]

[89] H J Almquist Physiol Rev 21 194 (1941)
[90] L F Fieser M Tishler and W L Sampson J Biol Chem 137 659 (1941)

TABLE I[90]

2 METHYL-3 ALKYL AND 3 β ALKENYL 1 4 NAPHTHOQUINONES

| Compound | Effective dose in γ |
|---|---|
| 2 Methyl 3 phytyl 1 4 naphthoquinone[91, 92] (Vitamin $K_1$) | 1 |
| 2 Methyl 3 difarnesyl 1 4 naphthoquinone[93, 94] (Vitamin $K_2$) | 1 6 |
| 2 Methyl 3 farnesyl 1 4 *naphthoquinone*[95, 96] | 5 |
| 2 Methyl 3 (β γ dihydrophytyl) 1 4 naphthoquinone[95, 97, 98] | 8 |
| 2 Methyl 3 geranyl 1 4 naphthoquinone[92] | 25 |
| 2 Methyl-3 cinnamyl 1 4 naphthoquinone[99] | 25 |
| 2 Methyl 3 (β γ γ trimethyl allyl) 1 4 naphthoquinone[99] | 50 |
| 2 3 Dimethyl 1 4 naphthoquinone[100] | 50 |
| 2 Methyl-3 benzyl 1 4 naphthoquinone[99] | 200 |
| 2 Methyl 3 hydrocinnamyl 1 4-naphthoquinone[97] | 300 |
| 2 Methyl 3 octadecyl 1 4 naphthoquinone[98, 100] | Inactive at 1000 |

TABLE II[101]

2 ALKYL AND 2 β ALKENYL 1 4 NAPHTHOQUINONES

| Compound | Effective dose in γ |
|---|---|
| 2 Methyl 1 4 naphthoquinone[102] | 0 3 |
| 2 Phytyl 1 4 naphthoquinone[96, 103, 104] | 50 |
| 2 Farnesyl 1 4 naphthoquinone[96, 103] | 500 |
| *2 (β γ Dihydro phytyl) 1 4 naphthoquinone* | *600* |
| 2 *n* Hexadecyl 1 4 naphthoquinone[105] | More than 600 |
| 2 *n* Octadecyl 1 4 naphthoquinone[105] | More than 600 |
| 2 Allyl 1 4 naphthoquinone[106, 107] | 800 |
| 2 Geranyl 1 4 naphthoquinone[96, 103] | 1000 |
| 2 Ethyl 1 4 naphthoquinone[106, 108, 109] | Inactive at 1000 |
| 2 *n* Propyl 1 4 naphthoquinone[105, 108, 109] | Inactive at 1000 |

91 L F Fieser *J Am Chem Soc* 61 2[illegible]9 (1939)
92 L F Fieser *Ibid* 61 3467 (1939)
93 R W McKee S B Binkley D W MacCorquodale S A Thayer and E A Doisy *Ibid* 61 1295 (1939)
94 H Dam J Glavind and P Karrer *Helv Chim Acta* 23 2[illegible]4 (1930)
95 L F Fieser M Tishler and W I Sampson *J Am Chem Soc* 62 99[illegible] 1[illegible]8 (1940)
96 L F Fieser M Tishler and N L Wendler *Ibid* 62 [illegible]861 (1940)
97 *M Tishler L F Fieser and N L Wendler Ibid 62* [illegible]866 *(1940)*
98 P Karrer and A Epprecht *Helv Chim Acta* 23 27[illegible] (1940)
99 L F Fieser W P Campbell E M Frey and M D Gates *J Am Chem Soc* 61 [illegible] 3216 (1939)
100 E Fernholz S Ansbacher and H B MacPhillamy *Ibid* 62 430 (1940)
101 L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 659 (1941)
102 S Ansbacher and E Fernholz *J Am Chem Soc* 61 19[illegible]4 (1939)
103 L F Fieser M Tishler and W L Sampson *Ibid* 62 996 1628 (1940)
104 H Dam J Glavind and P Karrer *Helv Chim Acta* 23 2[illegible]4 (1940)
105 E Fernholz S Ansbacher and H B MacPhillamy *J Am Chem Soc* 62 430 (1940)
106 L F Fieser W P Campbell and F M Frey *Ibid* 61 [illegible]206 (1939)
107 L F Fieser D M Bowen W P Campbell M Fieser E M Frey R N Jones B Riegel C E Schweitzer and P G Smith *Ibid* 61 19[illegible] (1939)
108 M Tishler and W I Sampson *Ibid* 61 563 (1939)
109 B Sjogren *Z physiol Chem* 264 1 (1939)

TABLE III[110]

HIGHLY ALKYLATED NAPHTHOQUINONES

| Compound | Effective dose in γ |
|---|---|
| 2 Ethyl 3 phytyl 1 4 naphthoquinone[111, 112] | 1000 |
| 2 3 Diallyl 1 4 naphthoquinone[113, 114] | 1000 |
| 2 5 Dimethyl 1 4 naphthoquinone[115] | 500 |
| 2 6 Dimethyl 1 4 naphthoquinone | Inactive at 1000 |
| 2 7 Dimethyl 1 4 naphthoquinone | 1000 |
| 2 8 Dimethyl 1 4 naphthoquinone[115] | 500 |
| 6 7 Dimethyl 1 4 naphthoquinone[113, 114] | Inactive at 1000 |
| 2 6 Dimethyl 3 phytyl 1 4 naphthoquinone[116] | Inactive at 1000 |
| 1 1 Dimethyl 3 *tert* butyl 1 4-dihydro anthraquinone[117] | Inactive at 1000 |
| 2 (δ Methyl γ pentenyl) 1 4 dihydro anthraquinone[117] | Inactive at 1000 |
| 1 2 4 Trihydroxy anthraquinone[118] | 100 |

TABLE IV[119]

CARBETHOXY AND HYDROXY NAPHTHOQUINONES

| Compound | Effective dose in γ |
|---|---|
| 2 Methyl-3-carbethoxy 1 4 naphthohydroquinone[120] | 25 |
| 2 Methyl 5 hydroxy 1 4 naphthoquinone[121] | 400 |
| 2 Methyl-3 hydroxy 1 4 naphthoquinone (Phthiocol)[122] | 500 |
| 5-Hydroxy 1 4 naphthoquinone (Juglone)[123] | Inactive at 1000 feebly active at 10 mg |
| 2 Hydroxy 1 4 naphthoquinone (Lawsone)[123, 124] | Inactive at 1000 active at 10 mg |
| 2 Hydroxy 3 dimethyl allyl 1 4 naphthoquinone (Lapachol)[123] | Inactive at 1000 active at 5 mg |
| 2 β Heptenyl 3 hydroxy 1 4 naphthoquinone | Inactive at 1000 |
| 2 Farnesyl-3 hydroxy 1 4 naphthoquinone[125] | Inactive at 1000 |
| 2 Methyl 3 (γ hydroxy-dihydrophytyl) 1 4 naphthoquinone[125] | Inactive at 1000 |
| Hydroquinone-diacetate[126] | Inactive at 1000 |

110 L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 659 (1941)
111 L F Fieser *J Am Chem Soc* 61 2559 (1939)
112 L F Fieser *Ibid* 61 3467 (1939)
113 L F Fieser W P Campbell and E M Fry *Ibid* 61 2206 (1939)
114 L F Fieser D M Bowen W P Campbell E M Frey and M D Gates *Ibid* 61 1926 (1939)
115 M Tishler L F Fieser and N L Wendler *Ibid* 62 1866 (1940)
116 L F Fieser *Ibid* 61 3467 (1939)
117 L F Fieser and C W Riegh rd *Ibid* 62 153 (1940)
118 G J Martin and C F Fisch r *J Biol Chem* 137 169 (1941)
119 L F Fieser M Tishler and W L Sampson *Ibid* 137 659 (1941)
120 C F Koelsch nd D J Byers *J Am Chem Soc* 62 560 (1940)
121 L F Fieser and J T Dunn *Ibid* 58 572 (1936)
122 H J Almquist and A A Klose *Ibid* 61 1611 (1939)
123 R Kuhn K Wallenfels F Weygand T Moll and L Hepding *Naturwissensch fte* 27 518 (1939)
124 H Dam J Glavind and P Karrer *Helv Chim Acta* 23 224 (1940)
125 M Tishler L F Fieser and N L Wendler *J Am Chem Soc* 62 2866 (1940)
126 M Tishler L F Fieser and N L Wendler *Ibid* 62 1982 (1940)

TABLE V[127]

NAPHTHOQUINONE OXIDES

| Compound | Effective dose in γ |
|---|---|
| Vitamin $K_1$ oxide[128, 129] | 1 2 |
| 2 Methyl 1 4-naphthoquinone oxide[130, 131] | 5 |
| 2,3 Dimethyl 1 4 naphthoquinone oxide[128, 129] | 25 |
| 2 Methyl-3-cinnamyl 1 4 naphthoquinone oxide[128, 129] | 150 |
| 2 Phytyl 1 4 naphthoquinone oxide[128, 129] | 200 |
| 2 Farnesyl 1 4 naphthoquinone oxide[128, 129] | 1000 |
| 2 7 Dimethyl 1 4 naphthoquinone oxide[130] | Inactive at 1000 |

TABLE VI[132]

MISCELLANEOUS QUINONES

| Compound | Effective dose in γ |
|---|---|
| 2 3 5-Trimethyl 1 4 benzoquinone | Inactive at 1000 |
| Duroquinone[133] | 10000 |
| 2 3 5 Trimethyl 6 phytyl 1 4 benzoquinone[134, 135] | Inactive at 1000 |
| α Tocopherylquinone[136] | Inactive at 1000 |
| 9 Methyl perinaphthenone 7[136] | Inactive at 1000 |
| 2 Methyl 2 phytyl 2 3-dihydro 1,4 naphthoquinone[135, 137] | 50 |
| Naphthotocopherol[134, 137] | 500 |
| 2 5 Dimethyl benzoquinone (Phlorone)[138, 139] | 3000 |

TABLE VII[140]

ESTERS AND ETHERS OF HYDROQUINONES

| Compound | Effective dose in γ |
|---|---|
| Sodium 2 methyl 1 4 naphthohydroquinone diphosphate[141, 142, 143] | 0 5 |
| Sodium 2 methyl 1 4 naphthohydroquinone disulfate[141, 143] | 2 |
| Vitamin $K_1$ hydroquinone diphosphoric acid[141] | 50 |
| Sodium 2 3-dimethyl 1 4 naphthohydroquinone disulfate[141] | 500 |
| Potassium vitamin $K_1$ hydroquinone disulfate[141] | Inactive at 500 |
| Diacetate of 2 methyl 1 4 naphthohydroquinone[144, 145] | 1 |
| Dibenzoate of 2 methyl 1 4 naphthohydroquinone[144, 145] | 1 |
| Dimesitoate of 2 methyl 1 4 naphthohydroquinone[146, 147] | 300 |
| Monoethyl ether of 2 methyl 1 4 naphthohydroquinone[148] | 1 |
| Dimethyl ether of 2 methyl 1 4 naphthohydroquinone[145] | 5 |
| Dibenzyl ether of 2 methyl 1 4 naphthohydroquinone[144] | 7 |
| Vitamin $K_1$ hydroquinone diacetate[149] | 2 |
| Vitamin $K_2$ hydroquinone diacetate[149] | 3 2 |

(See footnotes on opposite page)

TABLE VIII

AMINO COMPOUNDS

| Compound | Effective dose in γ |
|---|---|
| 4 Amino-2 methyl 1 naphthol hydrochloride[150, 151, 152] | About 1 |
| 4 Amino-3 methyl 1 naphthol hydrochloride[151] | About 1 |
| 2 Methyl 1 naphthylamine[153, 154] | 5 |

TABLE IX[155]

HYDRIDES OF VITAMIN K AND OF METHYL NAPHTHOQUINONE

| Compound | Effective dose in γ |
|---|---|
| 5 8-Dihydro-vitamin $K_1$[156, 157] | 4 |
| β γ 5 6 7 8-Hexahydro-vitamin $K_1$[156, 158] | 1000 |
| 2 Methyl 5 8-dihydro 1 4 naphthohydroquinone[157] | 6 |
| 2 Methyl 5 8 9 10 tetrahydro-1 4 naphthoquinone[157] | 8 |
| 2 Methyl 5 6 7 8-tetrahydro-1 4 naphthoquinone[158] | 500 |

[127] L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 6ɔ9 (1941)
[128] L F Fieser M Tishler and W L Sampson *J Am Chem Soc* 62 996 1628 (1940)
[129] M Tishler L F Fieser and N L Wendler *Ibid* 62 2866 (1940)
[130] L F Fieser W P Campbell E M Frey and M D Gates *Ib d* 61 2 ɔɔ 3 16 (1939)
[131] J Madinaveitia *Anales soc españ fis quim* 31 750 (1933)
[132] L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 6ɔ9 (1941)
[133] G J Martin and C F Lischer *Ibid* 137 169 (1941)
[134] L F Fieser M Tishler and W L Sampson *J Am Chem Soc* 62 ɔɔ6 1628 (1940)
[135] L F Fieser M Tishler and N L Wendler *Ibid* 62 2861 (1940)
[136] M Tishler L F Fieser and N L Wendler *Ibid* 62 2866 (1940)
[137] M Tishler L F Fieser and N L Wendler *Ib d* 62 198? (1940)
[138] S Ansbacher and E Fernholz *J Biol Chem* 131 399 (1939)
[139] H J Almquist *Physiol Rev* 21 194 (1941)
[140] L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 6 9 (1941)
[141] L F Fieser and E M Frey *J Am Chem Soc* 62 228 (1940)
[142] R H K Foster J Lee and U V Solmssen *Ibid* 62 453 (1940)
[143] S Ansbacher E Fernholz and M A Dolliver *Proc Soc Exptl Biol Med* 43 652 (1940)
[144] L F Fieser W P Campbell E M Frey and M D Gates *J Am Chem Soc* 61 2559 3216 (1939)
[145] S Ansbacher E Fernholz and M A Dolliver *Ibid* 62 155 (1940)
[146] M Tishler L F Fie er and N L Wendler *Ibid* 62 ?866 (1940)
[147] M Tishler L F Fie er and W L Sampson *Ibid* 62 1881 (1940)
[148] M Tishler L F Fie r and N L Wendler *Ibid* 62 198ɔ (1940)
[149] S B Binkl y D W MacCorquodale L C Cheney S A Thayer R W McKee and E A Doi y *Ib d* 61 1612 (1939)
[150] E A Doi y D W M cCorquodale S A Thayer S B Binkley and R W McKee *Science* 90 407 (1939)
[151] H J Almquist and A A Klose *Proc Soc Exptl Biol Med* 45 55 (1940)
[152] D Richt rt S A Thayer R W McKe S B Binkley and E A Doisy *Ib d* 44 601 (1940)
[153] M Tishl r L F Fieser and N L Wendler *J Am Chem Soc* 62 866 (1940)
[154] M Tishl r L F F eser and W L S mpson *Ib d* 62 1881 (1940)
[155] L F F eser M Ti hler and W L Sampson *J Biol Chem* 137 6ɔ9 (1941)
[156] L F Fies r M Ti hler and W L Sampson *J Am Ch m Soc* 62 996 1628 (1940)
[157] L F F eser M Tishler and N L Wendler *Ibid* 62 2861 (1940)
[158] M Ti hler L F Fieser and N L Wendler *Ibid* 62 2866 (1940)

TABLE X[159]

METHYL NAPHTHOLS METHYL TETRALONES AND RELATED COMPOUNDS

| Compound | Effective dose in γ |
|---|---|
| 2 Methyl 1 4 naphthohydroquinone | 0 5 |
| 2 Methyl 1 naphthol[160, 161] | 1 |
| 3 Methyl 1 naphthol[160, 161] | 0 6 |
| 4 Methyl 1 naphthol[160, 161] | Inactive at 1000 |
| 1 Methyl 2 naphthol[160, 161] | Inactive at 1000 |
| 3 Methyl 2 naphthol[160, 161] | Inactive at 1000 |
| 1 Naphthol | 1000 |
| 2 Methyl 1 tetralone[160, 161] | 0 6 |
| 3 Methyl 1 tetralone[160, 161] | 1 |
| β Methyl naphthalene | 1000 |

## 12 Determination

### (a) Physical Methods

**Spectroscopic Examination** The vitamins $K_1$ and $K_2$ and 2 methyl 1,4 naphthoquinone can be estimated by means of their absorption spectra,[16] provided the compounds are essentially free from other ab sorbing materials and are not present in the reduced hydroquinone form For a description of the spectra see page 485

### (b) Chemical Methods

**Colorimetric Redox Method**[163, 164] This method involves a cata lytic hydrogenation of the vitamin K quinone to the hydroquinone stage using butanol as the solvent and phenosafranin as the indicator An excess of 2 6 dichlorophenol indophenol is added to the reduced material in the absence of air The decrease in color is determined and this deter mination is a measure of the quinone originally present

This method is of course, not very specific since all quinones present will give the same reaction Highly colored solutions cannot be used but, after reduction the hydroquinone can be extracted with Claisen s alkali yielding colorless solutions which can then be used in this test

[159] L F Fieser M Tishler and W L Sampson *J Biol Chem* 137 659 (1941)
[160] M Tishler L F Fieser and N L Wendler *J Am Chem Soc* 62 2866 (1940)
[161] M Tishler L F Fieser and W L Sampson *Ibid* 62 1881 (1940)
[162] J L Pinder and J H Singer *Analyst* 65 7 (1940)
[163] J V Scudi *Proc Am Physiol Soc* 1941 252
[164] N R Trenner and F A Bacher *J Biol Chem* 137 745 (1941)

**Reaction with Sodium Alcoholates**[165, 166] The natural vitamins K give a purple blue color reaction with sodium ethylate and sodium methylate The color is unstable and turns into red and finally into brown During this reaction the vitamins are degradated to 2 methyl 3 hydroxy 1 4 naphthoquinone (phthiocol)[167] This color reaction is specific for those 1 4 naphthoquinone derivatives which have $\beta$ unsaturated side chains in the 3 position Carotenoids which may hinder the quantitative determination of the vitamin may be removed when the color has reached the red brown stage by extraction with a hydrocarbon solvent The color developed from the vitamin K remains in the alcohol phase

The sensitivity of this color reaction can be increased by testing the 2 4 dinitro phenyl hydrazones of the vitamins instead of the free vitamin[168] For this purpose an alcoholic solution of the vitamin (quinone) is mixed with a solution of 2 4 dinitro phenyl hydrazine in diluted hydrochloric acid and is gently heated A bluish green color is then developed with sodium methylate or a green color can be produced with ammonia and amyl alcohol The colors produced are stable

**Titanous Chloride Titration**[169] Vitamins K as quinones can be reduced quantitatively to the corresponding hydroquinones by means of titanous chloride the end point being shown by the use of an internal oxidation reduction indicator for example potassium indigo disulfonate or phenosafranin The determination is carried out under carbon dioxide in alcohol acetic acid solution in the presence of sodium carbonate and sodium potassium tartrate (Rochelle salt) in order to prevent the solution from becoming too acid during the titration

**Ethyl cyano-acetate Method**[169, 170] Vitamin K in alcoholic solution containing ammonia is mixed with ethyl cyano acetate followed by the addition of alkali A yellow color develops which is measured in a photometer The same reagent without the addition of alkali gives a violet color which however is too unstable to be useful

### (c) *Biological Methods*

There are two important biological methods for the determination of vitamins K Both of these methods measure in principle the clotting power of blood

[165] P Karrer *Helv Chim Acta* 22 114[illegible] (193[illegible])
[166] H J Almquist and A A Klose *J Am Chem Soc* 61 1611 [illegible] (1939)
[167] I F Fieser W P Campbell and E M Fry *Ibid* 61 [illegible] (1939)
[168] A Novelli *Science* 93 358 (1941)
[169] J I Pinde and J H Singer *Analyst* 65 7 (1940)
[170] R C[illegible]en *J Chem Soc* 1931 160

1 **Spontaneous Blood Clotting Time Determination** In the perfected form[171] of this assay procedure day old chicks are kept on a vitamin K deficient diet, care being taken to prevent coprophagy Within about 15 days severe avitaminosis develops The determination of the blood clotting time is then carried out by puncturing a wing vein and placing a tube containing the blood in a thermostat which is subjected to continuous shaking The clotting time is then defined as the time necessary *to form* a solid clot While the coagulation time of the blood of normal chicks is about 4–6 minutes, the blood of avitaminotic birds *may not coagulate for* several hours The determination of the vitamin K efficacy can be carried out according to this method, *using either a prophylactic or a curative* procedure The latter is usually more reliable After oral ingestion of *the vitamin preparation or after injection of the material to be tested* the blood clotting time is determined after six hours[171] or in a modified pro cedure *after 18 hours* [17] Avitaminotic birds which have previously been bled may show a decrease in the clotting time upon repeated bleeding several hours later without treatment with antihemorrhagic substances[173] and therefore should not be used in actual vitamin K assays

*2* **Prothrombin Clotting Time Determination** In this test the actual amount of prothrombin present in blood is determined[174] [17] and therefore the test is independent of various other factors which may affect the clotting time [176] In the most convenient form of this test[177] the ability of chicken blood to form clots in a minimum of time is determined The blood is obtained by decapitation or from the carotid artery and is run into a tube containing sodium oxalate solution Thromboplastin is prepared from chicken breast muscle[178] or from rabbit brain and then a calcium chloride solution is added The time from the addition of the calcium chloride until a firm clot is formed is measured Blood of normal birds clots within 25–30 seconds

*Many variations of this procedure* have been suggested and in practice each laboratory uses its own modified technic A practical important modification[178] is to dilute the blood plasma with an equal amount of Ringer solution and to mix this solution with thromboplastin extracts of

[171] S Ansbacher *J Nutrition* 17 303 (1937)
[172] S A Thayer R W McKee S B Binkley D W MacCorquodale and E A Doisy *Proc Soc Expll Biol Med* 40 478 (1939) 41 194 (1939)
[173] G Cheney *J Lab Clin Med* 24 919 (1939)
[174] A J Quick *Am J Physiol* 118 260 (1937)
[175] A J Quick *J Biol Chem* 34 LXXVIII (1940)
[176] R T Tidrick E T Joyce and H P Smith *Proc Soc Expll Biol Med* 42 853 (1939)
H J Almquist and A A Klose *Biochem J* 33 105 (1939)
H Dam and J Glavind *Ibid* 32 1018 (1938)

different concentrations in a series of parallel experiments The concentration which produces blood clotting in a defined length of time for example, within three minutes, is a measure of the amount of prothrombin present

In the curative procedure the effect of vitamin K may be determined after one[179] or four days While as pointed out above severe bleeding may reduce the whole blood clotting time no such effect has been observed on the prothrombin time [180]

Besides chicks, rabbits have also been recommended for use in this assay procedure [181] A photoelectric apparatus has also been devised for registering the clotting time [182]

The symptoms of a vitamin K deficiency can be obtained besides by dietary means by intoxication with chloroform[183] or with *p* toluene diamine [184]

## 13 Standards

No nationally or internationally accepted standard of vitamin K has been adopted so far Both 2 methyl 1 4 naphthoquinone[184, 185] and 2 methyl 1,4 naphthohydroquinone diacetate[186, 187] have been proposed as a reference standard since they can be obtained easily in pure form, can be characterized by physical constants and are relatively stable

The efficacy of various vitamin K preparations has been expressed in different units In the table[188] given below these various units are correlated The relation of these units to vitamin $K_1$ and to 2 methyl 1,4 naphthoquinone is given by the Thayer Doisy Unit which defines[189] the activity of 1 mg of vitamin $K_1$ as 1000 Thayer Doisy Units The efficacy of 2 methyl 1 4 naphthoquinone is about 3 3 times as great as that of vitamin $K_1$

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[179] A J Quick *J Biol Chem* 133 78 (1940)

[180] M C Elliott B Isaacs and A C Ivy *Proc Soc Exptl Biol Med* 43 40 (1940)

[181] P Meunier H Hinglais D Bovet and A Dreyfus *Compt rend* 210 454 (1940)

[182] K K Nygaard *J Lab Clin Med* 24 517 (1939)

[183] H P Smith E D Warner and K M Brinkhous *J Exptl Med* 66 803 (1937)

[184] S A Thayer S B Binkley D W MacCorquodale E A Doisy A D Emmett R A Brown and O D Bird *J Am Chem Soc* 61 2563 (1939)

[185] E Fernholz S Ansbacher and H B MacPhillamy *Ibid* 62 430 (1940)

[186] H Dam J Glavind and P Karrer *Helv Chim Acta* 23 224 (1940)

[187] D T Ewing J M Vandenbelt and O Kamm *J Biol Chem* 131 345 (1939)

[188] S Ansbacher *Proc Am Soc Biol Chem* 1940 111

[189] S A Thayer S B Binkley D W MacCorquodale E A Doisy A D Emmett R A Brown and O D Bird *J Am Chem Soc* 61 2563 (1939)

1 Thayer Doisy Unit[189] = 0 5 Ansbacher Unit [190]
= 0 5 Thayer Unit (1938) [191]
= 10 Dam Units [192]
= 0 25 Dann (1938) Unit [193]
= 0 625 Dann (1939) Unit [194]
= 0 08 ml Almquist Reference Standard

## 14 Physiology of Plants and Microorganisms

Plants and many microorganisms produce relatively large amounts of vitamins K This suggests that this vitamin is needed *for the maintenance* of life, and has actually been found to be a growth factor for Johne s bacillus [19] No information is available which would indicate the action of this compound in plants

## 15 Animal Physiology

### (a) *Metabolism of Vitamins K*

Vitamin K occurs in the intestinal tract from the daily food intake and from synthesis by microorganisms in the bowels Since the natural vitamins K are fat soluble compounds, they are absorbed only in the presence of bile salts [196] [197] especially desoxy cholic acid [198] Thus, in rats with bile fistula vitamin K deficiency occurs even when large doses are given orally unless bile salts are administered simultaneously Besides the presence of sufficient amounts of bile normal digestion of fats and apparently also a proper functioning of the liver[199] are necessary for vitamin K absorption Intestinal lesions interfere with vitamin K absorption The feeding of high doses of mineral oil together with vitamin K prohibits the normal absorption [200] This effect is not noted when the vitamin is injected Vitamins K are generally active upon parenteral injection Water soluble forms of vitamin K are absorbed from the intestinal tract

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[190] S Ansbacher *Proc Soc Exptl Biol Med* 44 248 (1940)
[191] S A Thayer D W McCorquodale R W McKee and E A Doisy *J Biol Chem* 123 CXX (1938)
[192] H Dam and J Glavind *Z Vitaminforsch* 9 71 (1939)
[193] F P Dann *Am J Physiol* 123 48 (1938)
[194] F P Dann *Proc Soc Exptl Biol Med* 42 663 (1939)
[195] D W Woolley and J R McCarter *Ibid* 45 357 (1940)
[196] J D Greaves and C L A Schmidt *Ibid* 37 43 (1937)
[197] H R Butt *Am J Digestive Diseases Nutrition* 6 127 (1939) H R Butt A M Snell and A E Osterberg *Proc Staff Meetings Mayo Clinic* 13 74 (1938)
[198] C L A Schmidt *Pacific Coast Med* 5 7 (1938)
[199] R L Clark C F Dixon H R Butt and A M Snell *Proc Staff Meetings Mayo Clinic* 14 407 (1939)
[200] M C Elliott B Isaacs and A C Ivy *Proc Soc Exptl Biol Med* 43 240 (1940)

even in the absence of bile salts[201, 202] and are especially useful for intravenous injections

Vitamins K are found only in very small amounts in blood (apparently only during times of transport) but in somewhat larger amounts in livers The animal organism has apparently no storage organs for this vitamin and metabolizes this compound fairly rapidly The mechanism of this metabolism is unknown No vitamin K is excreted through the kidneys[203], but considerable amounts are found in feces mainly due to the bacterial synthesis in the intestinal tract

Vitamin K is secreted in small but definite amounts in milk and eggs[204] There is apparently a special mechanism which regulates the passage of this vitamin through the placenta It has generally been observed that pregnant women just prior to parturition have an increased need for vitamin K The newborn infant usually has a slight K hypovitaminosis which can be overcome by feeding vitamin K to the infant or by giving it to the mother before delivery Better results are usually obtained by maternal administration than by giving vitamin K after birth[205]

### (*b*) *Physiological Action of Vitamins K*

Vitamins K are necessary for the maintenance of normal blood coagulation According to the classical theory[206] two different phases are involved in this process of coagulation The end effect which constitutes the second phase of the total process is the transformation of fibrinogen a protein dissolved in the blood plasma, into fibrin, a solid and essentially insoluble protein derivative This precipitation reaction is carried out by a postulated enzyme, the thrombin All experimental evidences point to the actual existence of this compound Thrombin does not occur in the blood, since otherwise the blood would not be liquid But the precursor of thrombin, namely prothrombin is present in plasma The first phase of the blood coagulation therefore, is the conversion of prothrombin into thrombin This process involves the action of another enzyme thromboplastin (or thrombokinase) in the presence of calcium ions While the latter are present in blood plasma thromboplastin is a normal cell constituent but not a blood constituent

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201 E D Warner K M Brinkhous and H P Smith *Proc Soc Exptl Biol Med* 44 607 (1940)

202 H P Smith and C A Owen *J Biol Chem* 134 783 (1940)

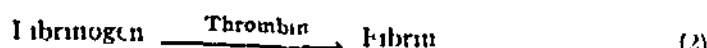
203 H Dam A Geiger J Glavind P Karrer W Karrer E Rothschild and H Salomon *Helv Chim Acta* 22 310 (1939)

204 H J Almquist and E L R Stokstad *J Nutrition* 12 329 (1936)

205 L B Shettles E Delfs and L M Hellman *Bull Johns Hopkins Hosp* 65 419 (1939)

206 O Hammarsten *Z physiol Chem* 28 98 (1899) P Morawitz *Biochem Z* 18 30 (1909)




During vitamin K deficiency only the prothrombin concentration is altered, that is, reduced [207] Vitamin K however is not identical with prothrombin since this vitamin does not cause blood coagulation *in vitro* [208] Vitamin K,[207] apparently, is also not a constituent of prothrombin since prothrombin fractions exert practically no vitamin K action[209] Since however, vitamin K influences the prothrombin concentration it is assumed that vitamin K is involved in the prothrombin formation This synthesis is apparently accomplished in the liver,[209, 210] since partial extirpation of the liver markedly reduces the prothrombin level in blood[211, 212] and since symptoms of a vitamin K deficiency even in the presence of normally adequate amounts of this vitamin have been observed frequently in cases of liver damages such as cirrhosis, hepatitis, atrophy and carcinoma [213] In all these cases the prothrombin concentration could not be influenced by vitamin K when given orally or injected intravenously or intramuscularly That a special mechanism for the formation of prothrombin actually exists is furthermore suggested by the fact that excessive feeding of vitamins K will not cause the prothrombin to rise above a certain level [214]

The mechanism of the vitamin K action is unknown It has been suggested [15] that the quinone structure of the vitamin takes part in an oxidation reduction system involving the oxidation of SH groups to —S—S linkages The hypothesis has been advanced[216] that the formation of the blood clot also involves an oxidation of SH groups of fibrinogen to —S—S groups in fibrin

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[207] H Dam *Nature* 135 652 (1935) F Schønheyder *Ibid* 135 652 (1935) H Dam F Schønheyder and E Tage Hansen *Biochem J* 30 1075 (1936) F Schønheyder *Ibid* 30 890 (1936)

[208] H Dam J Glavind L Lewis and E Tage Hansen *Skand Arch Physiol* 79 121 (1938)

[209] C L A Schmidt *Pacific Coast Med* 5 7 (1938)

[210] A M Snell *J Am Med Assoc* 112 1457 (1939)

[211] W D Andrus J W Lord and R A Moore *Surgery* 6 899 (1939)

[212] E D Warner *J Exptl Med* 68 831 (1938)

[213] P M Aggeler S P Lucia and L Goldman *Proc Soc Exptl Biol Med* 43 689 (1940) W D Andrus and J W Lord *J Am Med Assoc* 114 1336 (1940) H R Butt A M Snell A E Osterberg and J L Bollman *Proc Staff Meetings Mayo Clinic* 15 69 (1940) F J Pohle and J K Stewart *J Clin Investigation* 19 365 (1940) G H Scanlon K M Brinkhous W D Warner H P Smith and J E Flynn *J Am Med Assoc* 112 1898 (1939)

[214] A J Quick *Proc Am Soc Biol Chem* 34 LXXVIII (1940)

[215] F Bernheim and M L C Bernheim *J Biol Chem* 134 457 (1940)

[216] J P Baumberger *Proc Am Physiol Soc* 1941, 18

## 16 Hypovitaminosis and Avitaminosis

In vitamin K deficiency the prothrombin content of blood is markedly decreased and the blood clotting time is considerably prolonged The principle symptom during times of vitamin K deficiency is, besides a prolonged bleeding time, the occurrence of hemorrhages In chicks subcutaneous and intramuscular hemorrhages occur on the head neck, breast abdomen, back, wings and legs [17] In man hemmorrhagic syndromes caused by a vitamin K deficiency are frequently observed in an infant during the first few days of life when an extremely low prothrombin concentration generally is observed which lasts until a normal intestinal flora has been developed by the ingestion of food [18, 219, 220, 21] but they may also occur in adults [2] Low prothrombin levels are furthermore generally observed in humans when the absorption of the vitamin is impaired, such as in cases of obstructive jaundice [23, 224, 225] sprue [226] biliary fistula,[227, 228] and ulcerative colitis Hemophilia and thrombopenia however are not due to vitamin K deficiency and cannot be influenced by the administration of this vitamin [229, 30, 31]

### (a) Clinical Test Methods

1 Bleeding Time Determination A stab is made in the tip of a finger and the amount of time which it takes to stop the bleeding is observed In order to obtain measurable figures it has been suggested that the blood be absorbed every 15 seconds on a piece of filter paper The

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[217] S Ansbacher *J Nutrition* 17 303 (1937)

[218] G D Johnson *J South Carolina Med Assoc* 36 336 (1940)

[219] H Dam *et al* *Lancet* 2 1157 (1939)

[220] W W Waddell and D Guerry *J Pediatrics* 15 802 (1939)

[221] A J Quick and A M Grossman *Am J Med Sci* 199 1 (1940) *Proc Soc Exptl Biol Med* 40 647 (1939) 41 227 (1939)

[222] R Kark and E L Lozner *Lancet* 2 1162 (1939)

[223] S T Townsend and E S Mills *Can Med Assoc J* 41 111 (1939) 42 541 (1940)

[224] K M Brinkhous H P Smith and E D Warner *Am J Med Sci* 196 50 (1938) E D Warner K M Brinkhous and H P Smith *Proc Soc Exptl Biol Med* 37 628 (1938) A M Snell T B Magath E W Boland A E Osterberg H R Butt J L Bollman and W Walters *Proc Staff Meetings Mayo Clinic* 13 65 (1938)

[225] A J Quick M Stanley Brown and F W Bancroft *Am J Med Sci* 190 501 (1935)

[226] P M Aggeler S P Lucia and L Goldman *Proc Soc Exptl Biol Med* 43 689 (1940) R L Clark C F Dixon H R Butt and A M Snell *Proc Staff Meetings Mayo Clinic* 14 407 (1939) R Engel *Med Welt* 13 120 (1939) H Hult *Nord Med* 3 2428 (1939)

[227] I C Zuckerman B Kogut M Jacobi and J Y Cohen *Am J Digest Dis* 6 337 (1939)

[228] L K Ferguson and D G Calder *Ibid* 6 722 (1939) H P Smith S E Ziffern C A Owen G R Hoffman and J E Flynn *J Iowa State Med Soc* 29 377 (1939)

[229] H Dam and J Glavind *Ugeskrift Laeger* 100 248 (1938)

[230] H Dam F Schønheyder and E Tage Jansen *Biochem J* 30 1075 (1936)

[231] G H Scanlon K M Brinkhous E D Warner H P Smith and J E Flynn *J Am Med Assoc* 112 1898 (1939)

blood drops should become continually smaller The bleeding normally stops after 1–3 minutes Continued bleeding may be due to many causes such as deficiency in vitamins C or K hemophilia etc

2 **Coagulation Time Determination** About 1 cc of fresh blood is drawn in a tube containing thromboplastin The tube is shaken and the clotting time is observed The determination is carried out with the blood of a normal person as a control This test[232] has been recommended[233] as a bedside method since it is easy to carry out and is very reliable

3 **Prothrombin Time Determination** This method has already been described among the methods used for the determination of vitamin K and has been most successfully used clinically in various modifications[233, 234, 235, 236] Various micromethods have been worked out[237] which require only one drop of blood

4 **Two-Stage Determination of Prothrombin** In this test[238] the blood is first mixed with a sodium oxalate solution and is centrifuged The plasma is then defibrinated by adding thrombin The remaining fluid contains the prothrombin which is completely converted into thrombin by mixing various dilutions with exactly defined solutions of saline, calcium ions and thromboplastin A specified amount of fibrinogen is then added and the amount of thrombin previously formed is measured by determination of the clotting time

5 **Serum Volume Test**[239] The volume of the blood serum adjusted to the red cell count, decreases during early stages of vitamin K hypovitaminosis and its determination in conjunction with an examination of the blood clot for friability has been claimed to serve as an indication of danger of bleeding

## 17 Hypervitaminosis

The naturally occurring vitamins $K_1$ and $K_2$ are non toxic, even when given in excessive doses [40] Thus in mice no lethal effect could be pro

[232] H P Smith S L Ziffern C A Owen and G R Hoffman *J Am Med Assoc* 113 380 (1939) S E Ziffern C A Owen G R Hoffman and H P Smith *Proc Soc Exptl Biol Med* 40 595 (1939)

[233] H R Butt and A M Snell *Vitamin K* Philadelphia 1941

[234] A J Quick M Stanley Brown and F W Bancroft *Am J Med Sci* 190 501 (1935)

[235] A J Quick *Am J Clin Path* 10 222 (1940)

[236] T B Magath *Am J Clin Path* (Tech Suppl ) 3 187 (1939)

[237] A J Quick *Proc Soc Exptl Biol Med* 42 788 (1939) W E Bray and O R Kelly *Am J Clin Path* 10 154 (1940) O R Kelly and W E Bray *J Lab Clin Med* 25 27 (1940) K Kato *Am J Clin Path* 10 147 (1940)

[238] E D Warner K M Brinkhous and H P Smith *Am J Physiol* 114 667 (1936)

[239] E F Boyce and E M McFetridge *New Orleans Med Surg J* 91 357 (1939)

[240] S Ansbacher *Proc Am Soc Biol Chem* 1940 III

duced with doses as high as 25 g of vitamin $K_1$ per kilogram of body weight[241] (studies on man[4]) The easily available substitutes 2 methyl 1 4 naphthoquinone and the 2 methyl 1 4 naphthohydroquinone however show definite toxic symptoms[43, 244] when fed to dogs rabbits or humans in excessive amounts Vomiting and porphyrinuria and occasionally albuminuria have been observed when 180 mg of these compounds was given orally to humans or when 30–60 mg per kilogram body weight was fed to dogs In rabbits excess feeding of 2 methyl 1,4 naphthoquinone has been noted to cause a prolongation of the blood clotting time which amounts to an effect which is contrary to that obtained by feeding normal doses of vitamin K[245] In mice small quantities of 2 methyl 1 4 naphthoquinone and similarly also phthiocol cause a drop in the erythrocyte count and hemoglobin while quantities from 0 2 to 0 5 g per kilogram body weight cause death[241]

## 18 Requirements

Vitamins K are required by all animals experimentally investigated such as the chick duck goose, canary, pigeon,[46] turkey,[47] rat[248, 249] rabbit,[250] mouse[251] dog[25] and man[23] The actual amount of this vitamin required by different species is unknown In human therapy amounts varying from 1 to 10 mg of 2 methyl 1 4 naphthoquinone have been recommended[254] In general pregnant and lactating women need increased amounts of this vitamin in order to protect the newborn from hypovitaminosis (See also page 614 )

Vitamins K are not required by most microorganisms such as yeast fungi and most bacteria but seem necessary for growth of Johne s bacillus[25]

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[241] H Molitor and H J Robinson *Proc Soc Exptl Biol Med* 43 1 (1940)
[*] H R Butt and A M Snell *Vitamin K* Philadelphia 1941
I Koller *Schweiz med Wochschr* 45 11 1 (1130)
H Molitor and H J Robinson *Proc Soc Exptl Biol Med* 43 1 (1940)
P Meunier H Hinglais D Bovet and A Dreyfus *Compt rend* 210 454 (1940)
[46] H Dam F Schønheyder and L Lewis *Biochem J* 31 22 (1937)
*H J Almquist Proc Seventh World's Poultry Congress Cleveland 1939 138*
H Dam and J Glavind *Z Vitaminforsch* 9 71 (1939)
[249] J D Greaves *Am J Physiol* 125 4 9 (1939)
[250] H Dam and J Glavind *Acta Med Scand* 96 108 (1938)
[251] R Murphy *Science* 89 203 (1939)
[252] W B Hawkins and K M Brinkhous *J Exptl Med* 63 795 (1936)
[253] R Kark and F L Lozner *Lancet* 237 1162 (1939)
[254] H Dam *Ann Rev Biochem* 9 373 (1940)
[255] D W Woolley and J R McCarter *Proc Soc Exptl Biol Med* 45 357 (1940)

# VITAMIN P

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# VITAMIN P

## 1 Nomenclature and Survey

Names

Vitamin P[1,2]
Citrin[1,2]
Permeability vitamin

Composition

Vitamin P is the name given to a crude extract which contains besides other physiologically active compounds the glucosides eriodictin and hesperidin which yield upon hydrolysis eriodictyol and hesperitin

## 2 Chronology

1936 ARMENTANÓ BENTSÁTH BÉRES RUSZNYÁK and SZENT GYÖRGYI[1,2] reported the occurrence of a substance other than vitamin C which controls hemorrhages in man The active material was identified as a flavanone

1939 SCARBOROUGH[3] presented evidence from experiments on human subjects which established the existence of a factor decreasing capillary fragility

## 3 Occurrence

The distribution of vitamin P in nature has not been investigated for a large number of foods It has been shown[4,5] that this vitamin is present in citrus fruits, such as lemon orange and grapefruit The skin of these fruits is generally richer than the juice from the pulp Lemon juice contains more vitamin P than does orange juice and this in turn contains more than grapefruit juice Vitamin P is also found in the juice of other plants such as paprika and is believed to be widely distributed over the entire plant kingdom

It has not been possible to demonstrate the presence of vitamin P (eriodictyol or hesperitin) in animal materials such as milk liver or kidney[6,7]

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[1] L Armentanó A Bentsáth T Béres and I Rusznyák *Deut med Wschr.*, 62 1326 (1936)
[2] A Bentsáth I Rusznyák and A Szent Györgyi *Nature* 138, 798 (1936)
[3] H Scarborough *Biochem J* 33 1400 (1939)
[4] I Rusznyák and A Szent Györgyi *Nature* 138, 27 (1936)
[5] A Szent Györgyi *Z physiol Chem* 255 126 (1938)
[6] I Robezmieks *Z Vitamins f rsch* 8 27 (1938–39)
[7] W Neuweiler *Ibid* 9 338 (1939)

Vitamin P appears to occur in plants predominantly in the form of glucosides[8]

## 4 Isolation

The isolation of vitamin P is based on its property of being precipitated by lead salts in neutral solution and by alkali in anhydrous alcoholic solution The actual isolation procedure, for example, from lemons, is carried out as follows[9] The ripe fruit is pressed and the peels are ground and extracted with 96% alcohol By the addition of Ba and Pb acetate solutions to the combined juice and extracts considerable amounts of impurities are precipitated The vitamin is then precipitated by neutralizing the solution with ammonium hydroxide The precipitate can be purified by reprecipitation and is then suspended in alcohol to which dilute sulfuric acid is added Lead sulfate precipitates, and the vitamin goes into solution The vitamin is obtained from the alcoholic solution by fractional precipitation with alkali The free vitamin is obtained from the alkali salt by acidification The procedure is essentially the same for the isolation of vitamin P from dried material but the operations are reversed After an initial alcohol extraction the vitamin is precipitated with alkali, the precipitate is dissolved in acetic acid and the vitamin is precipitated with lead acetate and ammonium hydroxide

Two final products are obtained a crystalline material consisting essentially of hesperidin with some eriodictin,[10] and the mother liquor which contains mainly eriodictin plus some hesperidin The crystalline material and the solution apparently contain other physiologically active components in addition to eriodictin and hesperidin An efficient method for the preparation of absolutely pure eriodictin or hesperidin has not been worked out and a separation has not been accomplished satisfactorily The crystalline material can be recrystallized from pyridine and water[11]

The glucosides eriodictin and hesperidin yield upon hydrolysis with dilute acids eriodictyol and hesperitin

## 5 Properties

The chemical and physical properties of vitamin P cannot be stated since the chemistry is not definitely established Concentrates contain eriodictin and hesperidin besides other unknown components

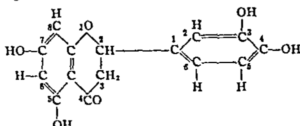
[8] V Bruckner and A Szent Györgyi *Nature* 138 1057 (1936)
[9] A Szent Györgyi *Z physiol Chem* 255 126 (1938)
[10] V Bruckner and A Szent Györgyi *Nature* 138 1057 (1936)
[11] H Scarborough *Biochem J* 33 1400 (1939)

Eriodictyol forms light yellow crystals which melt at 267° C. These crystals are sparingly soluble in water, but very soluble in organic solvents and in alkali.

Hesperitin melts at 224° C. and is readily soluble in water and in organic solvents.

## 6. Constitution

There are probably two glucosides which are vitamins P:[11] namely, *hesperidin*, which is hesperitin *l*-rhamnosido-*d*-glucose, and *eriodictin*, which is eriodictyol *l*-rhamnoside. Indications are that these compounds may be active only when the flavanone rings are opened to the corresponding chalcones. In the glucosides the sugar residues are attached to the 7-position. Hesperitin is the 4′-methyl ether of eriodictyol. The latter has the following constitution:




Eriodictyol
5,7,3′,4′-Tetrahydroxy flavanone

## 7. Biogenesis

The method which is used in the plant organism for the synthesis of vitamin P is not known. Various theories concerning the formation of flavanones in plants have been advanced.[12] The biogenetic scheme implies a synthesis from carbohydrates. Specifically, the flavanones appear to be built up from two hexoses and one triose by means of aldol condensations. Coniferyl alcohol, a compound which is believed to be a building unit of lignin, may be an intermediate.

## 8. Specificity

Little is known about the specificity of vitamin P. Besides the hydrolyzed vitamin concentrate containing eriodictyol and hesperitin, the naturally occurring glucosides have been shown to be active.

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[11] A. Mager, *Z. Phys. Chem.*, 274, 109 (1942). G. Zemplén and R. Bognár, *Ber.*, 76, 773 (1943). C. Z. Wawra and J. L. Webb, *Science*, 96, 302 (1942). R. H. Higby, *J. Am. Pharm. Assoc.*, 32, 74 (1942).

[12] R. Robinson, *Nature*, 137, 172 (1936).

## 9 Determination

### (a) *Chemical Methods*

There is no chemical method by which vitamin P can be detected This is due mainly to the fact that the chemical composition of vitamin P is not known Certain color reactions for the flavanones, such as the ones isolated from vitamin P preparations, cannot be accepted as tests for the vitamin as long as their vitamin nature is not better defined

Vitamin P is said to give a yellow color with boric acid [13] This color reaction may be used for the determination of flavones in natural products The flavanones, on the other hand, give no color reaction with boric acid Since vitamin P preparations contain predominantly flavanones (eriodictin and hesperidin), the positive color reaction must be due to some other unknown constituent

### (b) *Biological Methods*

The biological methods recommended for the demonstration of the presence of vitamin P are based on the fact that during vitamin P deficiency the capillary resistance of experimental animals is diminished The occurrence of hemorrhages (type and number) has also been used as a criterion for vitamin P deficiency but with less success [14,15,16] Guinea pigs have been used predominantly[17,18,19,20,21] as experimental animals but the rat responds equally well to a vitamin P deficient diet and can be healed by the administration of this vitamin

## 10 Standards

No standard of vitamin P has been designated

## 11 Physiology of Plants

The physiological reason for the presence of vitamin P in the plant organism is not known The flavanones may constitute structural building units

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[13] C W Wilson *J Am Chem Soc* 61 2303 (1939)
[14] A Szent Györgyi *Z physiol Chem* 255 126 (1938)
[15] S S Zilva *Biochem J* 31 915 1488 (1937)
[16] T Moll *Klin Wochschr* 16 1653 (1937)
[17] A Bentsáth I Rusznyák and A Szent Györgyi *Nature* 138 798 (1936)
[18] A Bentsáth I Rusznyák and A Szent Györgyi *Ibid* 139 326 (1937)
[19] E N Todhunter R C Robbins G Ivey and W Brewer *J Nutrition* 19 113 (1940)
[20] A Bentsáth and N B Das *Z physiol Chem* 247 258 (1937)
[21] C E Zacho *Acta Path Microbiol Scand* 16 1411 (1939)
[22] I Rusznyák and A Benko *Science* 94 2[illegible] (1941)

It has been suggested[23] that vitamin P acts in plants as a detoxifying agent and that considerable amounts of this agent are held available for times of need in the form of a glucoside This is feasible since certain other biologically important compounds are stored in the plant organism in the form of inactive glucosides and, when needed the glucosides are hydrolyzed and the biologically active compounds are set free

## 12 Animal Physiology

Vitamin P (and probably the glucosides after hydrolysis in the intestines) is absorbed from the intestinal tract Excess amounts are excreted through the urine [24, 25] Vitamin P is also active when given by intramuscular or intravenous injection or through the rectum The physiological action of vitamin P is concerned with the maintenance of normal conditions in the walls of the small blood vessels and the absence of this vitamin causes increased capillary fragility and permeability It has been suggested that the action of vitamin P in protecting capillary resistance may be due to a detoxifying power [26] The action of vitamin P, however, is not due to a change in concentration of any of the blood clotting factors such as prothrombin fibrinogen or platelets It has been demonstrated on the other hand, that vitamin P is able to increase the calcium level in the blood [27] The view has also been expressed that vitamin P may be required for the absorption and retention of ascorbic acid [28]

Vitamin P but apparently not the isolated constituents hesperidin and eriodictin causes upon intravenous injection a definite drop in blood pressure [29, 30] as demonstrated on the cat, rabbit frog and turtle [31]

Vitamin P also promotes growth and cell division in the fertilized ovum of sea urchins [32]

## 13 Avitaminosis

A state of vitamin P deficiency is known for the guinea pig rat and for the human organism In patients suffering from vitamin P deficiency

[23] I N Kugelmass *J Am Med Assoc* 115 519 (1940)
[24] I Armentanó E B Hatz and I Rusznyak *Klin Wochsch* 17 733 (1938)
[25] I Huszak *Z physiol Chem* 249 14 (1937)
[26] I N Kugelmass *J Am Med Assoc* 115 519 (1940)
[27] M Raunert *Z Urol* 32 630 (1938)
[28] A Elmby and E Warburg *Lancet* 1937 II 1363
[29] A Szent-Györgyi *Z physiol Chem* 255 126 (1938)
[30] I Armentanó *Z ges exp Med* 102 219 (1937)
[31] A J Leser C F Lombard C H Thienes C Wawra and J L Webb *Proc Am Soc Pharmacol Exptl Therap* 1941 26
[32] F Ludwig *Arch exp Path Pharmakol* 189 243 (1938)

capillary resistance is decreased and vascular permeability is increased The clinical symptoms comprise hemorrhagic conditions of the skin (nutritional purpura) which cannot be cured by vitamin C [33, 34, 35, 36, 37, 38] Vitamin P therapy has also been found of value in hemorrhagic conditions of otherwise non specific character, such as in cases of bleeding in the kidney (nephritis) and in the stomach [39]

## 14 Requirements

The exact vitamin P requirement of the human organism is not known In clinical cases 50–150 mg of the natural mixture of compounds have been administered Rats with diminished capillary resistance have been given 3–4 mg per day subcutaneously

[33] L Armentanó A Bentsáth T Béres and I Rusznyak *Deut med Wochschr* 62 1326 (1936)
[34] H Scarborough *Biochem J* 33 1400 (1939)
[35] I N Kugelmass *J Am Med Assoc* 115 519 (1940)
[36] S Lajos *Klin Wochschr* 16 1615 (1937)
[37] C T Decker *Münch med Wochschr* 86 292 (1939)
[38] T Jersild *Lancet* 1938 I 1445
[39] M Raunert *Z Urol* 32 630 (1938)

# NON-IDENTIFIED
# VITAMINS

# THE NON-IDENTIFIED VITAMINS

In addition to the vitamins which are identified and which have been discussed in the preceding chapters of this monograph, there is an unknown number of non identified vitamins Their existence has as a general rule been postulated since it was found that an animal species kept on a more or less purified diet ceased to grow or developed specific diseases which could be alleviated by the feeding of special foods As a result a great number of unknown vitamins have been claimed It remains to be seen how many of these dietary essentials will prove to be separate entities On the other hand there is the definite possibility that future research will uncover the existence of other vitamins the existence of which is unknown today

## 1 Vitamin $B_3$

In the early work on vitamin $B_1$ from 1911 to 1920 it was noted[1] that an additional factor (called vitamin $B_3$[2]) was necessary in order to cause pigeons to gain weight Vitamin $B_3$ was described by Williams and Waterman in 1928[3] A clearer definition of this vitamin was offered in 1936 by Carter and O Brien[4] Vitamin $B_3$ occurs in liver yeast whole grains and malt It is soluble in water and dilute alcohol and sensitive to heat and alkali Vitamin $B_3$ is present in the filtrate fraction after adsorption of an extract on fuller s earth[5] It is possible that vitamin $B_3$ is identical with pantothenic acid

## 2 Vitamin $B_4$

The existence of a special factor (or factors) designated as vitamin $B_4$ which prevents the occurrence of a typical paralysis in rats[6] and in chicks[7]

[1] H Schaumann *Trans Soc Tropical Med Hyg* 5 59 (1911) F A Cooper *J Hyg* 12 436 (1912) A D Emmett and L H McKim *J Biol Chem* 32 409 (1917)

[2] R R Williams and W H Eddy *Carnegie Inst Wash Year book* 27 378 (1928)
R R Williams and R F Waterman *J Biol Chem* 78 311 (1928)

[4] C. W Carter and J R O Brien *Biochem J* 30 43 (1936)

[5] C W Carter and J R O Brien *Ibid* 33 1810 (1939)

[6] V Reader *Biochem J* 23 689 (1929) O L Kline C A Elvehjem and F B Hart *Ibid* 30 780 (1936)

[7] J A Keenan O L Kline C. A Elvehjem and F B Hart *J Biol Chem* 103 671 (1933)

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I Schaumann *Trans Soc T p cal Med Hyg* 5 59 (1911) E A Cooper *J Hyg* 12 436
) A D Emmett and L H McKim *J Biol Chem* 32 409 (1917)
R Williams and W H Eddy *Carneg e Inst Wash Yea book* 27 378 (19 8)
L R Williams and R F Waterm n *J Bi l Chem* 78 311 (19 8)
– W Carter and J R O Brien *Biochem J* 30 43 (1936)
W Carter and J R O Brien *Ibid* 33 1810 (1939)
' Reader *Bioch m J* 23 689 (1929) O L Kl ne C A El hjem and E B Hart *Ibid* 30 780
)
A K enan O L Kl n C A El ehj m and E B H rt *J Biol Chem* 103 671 (1933)

has been assigned to this compound Adenine has been shown to be necessary for certain strains of lactic acid bacteria[17] and it has been claimed that adenylic acid is the coenzyme of the fatty acid dehydrogenase[18] In clinical studies[19] on pellagrins adenylic acid seemed to increase the effect of nicotinic acid When 3 to 20 mg of yeast adenylic acid were injected intravenously into patients deep involuntary, gasping inspiration a fluttering sensation in the upper part of the abdomen and a feeling of fullness in the head were observed

### 6 Vitamin $B_c$

The occurrence of anemia has been observed in chicks as the result of a deficiency of an unknown member of the vitamin B complex[20] The postulated vitamin is present in liver but has not been identified The deficiency syndrome comprises a decreased red blood cell count decreased per cent of hemoglobin in the blood and decreased red cell volume In addition to showing the symptoms of anemia young animals deficient in this vitamin grow only slowly

### 7 Vitamin $B_p$ (Anti-Perosis Vitamin)

Perosis a deficiency disease characterized by the occurrence of deformed legs and known also as hock disease or slipped tendon has frequently been observed in chicks on synthetic diets[21] The lower bones in perotic chicks are abnormally short and twisted Perosis can be treated to a certain extent prophylactically by manganese[2] and by choline[23] In addition there seems to exist another perosis preventing compound of organic chemical nature[24]

### 8 Vitamin J[25]

Vitamin J which has at times also been called vitamin $C_2$ has been postulated as an anti pneumonia factor The existence of such a factor has been

[17] E F Möller Z angew Chem 52 466 (1939)
[18] K. Lang and H Mayer Z physiol Chem 262 120 (1939)
[19] T D Spies D P Hightower and L H Hubbard J Am Med Assoc 115 292 (1940)
[20] A G Hogan and E M Parrott J Biol Chem 132 507 (1940)
[21] A. G Hogan N B Guerrant and H L Kempster Ibid 64 113 (19‥5) A. G Hogan and C L. Shrewsbury J Nutrition 3 39 (1930) A G Hogan L R Richardson and H Patrik Ibid 19 Proc 14 (1940)
[22] H. S Wilgus L C Norris and G F Heuser J Nutrition 14 1‥ (1937)
[23] T H Jukes J Biol Chem 134 78‥ (1940)
[24] A G Hogan L R. Richardson H Patrik and H L Kempster J Nutrition 21 327 (1941)
[25] H v Euler H Soder and M M lmberg Z Hyg Infekt Krankh 116 672 (1935)

has been postulated This factor is present in yeast and in liver Dried grass, wheat germ, pork brain and pork kidney are good sources while grains are relatively poor sources [8] Rats on a vitamin $B_4$ deficient diet show general muscular weakness, spastic gait, swollen paws and a tendency to sit in a hunched position [9] Chicks which are deficient in vitamin $B_4$ show a disturbed gait, a lack of coordination and a tendency to fall on their side with their legs in tension pulled against the abdomen It has been suggested[10] that vitamin $B_4$ may be identical with a mixture of arginine and glycine, since a deficiency of these amino acids causes the occurrence of symptoms in the chick which resemble closely those observed in vitamin $B_4$ deficiency

### 3 Vitamin $B_5$

The term vitamin $B_5$ was given in 1930[11] to a fuller s earth eluate fraction from yeast which was shown to be a factor essential for maintaining weight in pigeons Vitamin $B_5$ was found to be stable toward heat and alkali Later investigations have shown[12] that the vitamin $B_5$ concentrate contains vitamin $B_6$ which latter compound has weight maintaining properties for the pigeon, but the effects of which are less pronounced than those of the vitamin $B_5$ concentrate The remaining factor is now called vitamin $B_5$ It seems probable that vitamin $B_5$ is identical with nicotinic acid[12] since nicotinic acid exhibits the weight maintaining properties of vitamin $B_5$ and has been isolated from eluate fractions [13] It has been reported independently that nicotinic acid is required by the pigeon [14]

### 4 Vitamin $B_7$—Vitamin I

Vitamin $B_7$ or Vitamin I is a name given to a substance present in rice polishings which is soluble in methanol and in ethyl alcohol In the absence of this factor, pigeons develop digestive disturbances [15]

### 5 Vitamin $B_8$—Adenylic Acid

It has repeatedly been suggested[16] that adenylic acid (or its degradation product, adenine) might exert vitamin activity and the term vitamin $B_8$

8 O L Kline H R Bird C A Elvehjem and E B Hart *J Nutrition* 11 515 (1936) 12 455 (1936)
9 V Reader *Biochem J* 24 18 7 (1930)
10 D M Hegsted G M Briggs C A Elvehjem and E B Hart *J Biol Chem* 140 ?01 (1941)
11 C W Carter H W Kinnersly and R A Peters *Biochem J* 24 1832 1844 (1930)
12 C W Carter and J R O Brien *Ibid* 33 1810 (1939)
13 T F Macrae and C E Edgar *Ibid* 31 2225 (1937)
14 L J Harris *J Soc Chem Ind* 58 471 (1939)
15 E Centanni *Biochim terap sper* 22 137 (1935)
16 H v Euler F Schlenk L Melzer and B Högberg *Z phys ol Chem* 258 212 (1939)

has been assigned to this compound Adenine has been shown to be necessary for certain strains of lactic acid bacteria[17] and it has been claimed that adenylic acid is the coenzyme of the fatty acid dehydrogenase[18] In clinical studies[19] on pellagrins adenylic acid seemed to increase the effect of nicotinic acid When 3 to 20 mg of yeast adenylic acid were injected intravenously into patients deep involuntary, gasping inspiration, a fluttering sensation in the upper part of the abdomen and a feeling of fullness in the head were observed

## 6 Vitamin $B_c$

The occurrence of anemia has been observed in chicks as the result of a deficiency of an unknown member of the vitamin B complex[20] The postulated vitamin is present in liver but has not been identified The deficiency syndrome comprises a decreased red blood cell count decreased per cent of hemoglobin in the blood and decreased red cell volume In addition to showing the symptoms of anemia young animals deficient in this vitamin grow only slowly

## 7 Vitamin $B_p$ (Anti-Perosis Vitamin)

Perosis a deficiency disease characterized by the occurrence of deformed legs and known also as hock disease or slipped tendon has frequently been observed in chicks on synthetic diets[21] The lower bones in perotic chicks are abnormally short and twisted Perosis can be treated to a certain extent prophylactically by manganese[22] and by choline[23] In addition there seems to exist another perosis preventing compound of organic chemical nature[24]

## 8 Vitamin J[25]

Vitamin J which has at times also been called vitamin $C_2$ has been postulated as an anti pneumonia factor The existence of such a factor has been

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[17] E F Möller *Z angew Chem* 52 466 (1939)
[18] K Lang and H Mayer *Z physiol Chem* 262 120 (1939)
[19] T D Spies D P Hightower and L H Hubbard *J Am Med Assoc* 115 29? (1940)
[20] A G Hogan and E M Parrott *J B l Chem* 132 507 (1940)
[21] A. G Hogan N B Guerrant and H L Kempster *Ibid* 64 113 (1925) A G Hogan and C L Shrewsbury *J Nutrition* 3 39 (1930) A G Hogan L R Richardson and H Patrik *Ib d* 19 Proc 14 (1940)
[22] H S Wilgus I C Norris and G I H er *J Nutrition* 14 1? (1937)
[23] T H Jukes *J Biol Chem* 134 83 (1940)
[24] A G Hogan L R Richardson H Patrik and H L Kempster *J Nutrition* 21 327 (1941)
[25] H v Euler H Soder and M Malmberg *Z Hyg I fekti nskrankh* 116 67? (1935)

postulated because guinea pigs infected with pneumococci show remarkable resistance when lemon juice is administered Since it is known that vitamin C is present in lemon juice and since vitamin C is known to detoxify bacteria, the power of ascorbic acid for protecting guinea pigs against pneumonia was studied experimentally The effect of ascorbic acid was found to be considerably less than that of lemon juice It was therefore concluded that a special anti pneumonia factor exists in lemon juice As a further differentiation from vitamin C, vitamin J does not occur in paprika and in the eye lens In addition to lemons, it is also found in black currants rowanberries and elderberries The chemical nature of vitamin J is not known No studies have been published on the use of this vitamin in man

## 9 *Vitamins $L_1$ and $L_2$—Lactation Vitamins*

The existence of two different water soluble vitamins, necessary for the onset of normal lactation has been postulated [26] Vitamin $L_1$ is present in liver while vitamin $L_2$ is present in baker s yeast Neither of these factors can replace the other Much higher amounts of these vitamins are apparently necessary to induce the first lactation than are necessary for a second litter *The vitamins seem to be of functional importance in the* maturation of lactation tissue

Evidence for the existence of a factor essential for lactation has also been presented by another laboratory [27] The factor was found in rice polishings, defatted wheat embryo and brewer's yeast, but most abundantly in liver and in rice bran extracts

The factor or factors necessary for lactation appear to be different from all other known vitamins [26, 27, 28]

## 10 Vitamin M

The existence of a vitamin M [9] has been postulated, because monkeys kept on a diet supposedly containing all recognized food factors are known

[26] W Nakahara F Inukai and S Ugami *Science* 87 372 (1938) *Sc Papers Inst Phys Chem Research Tokyo* 31 42 (1937) 34 250 (1938) *Proc Imp Acad Tokyo* 14 9 (1938)

[27] B Sure *J Biol Chem* 140 CXXX (1941)

[28] W Nakahara F Inukai and S Ugam *Science* 91 431 (1940)

[29] P L Day W C Langston and W J Darby *Proc Soc Exptl Biol Med* 38 860 (1938) W C Langston W J Darby C F Shukers and P L Day *J Exptl Med* 68 923 (1938) P L Day W C Langston W J Darby J G Wahlin and V N ms *Ibid* 72 463 (1940) M Janota and G M Da k *J Infectious Diseases* 65 217 (1939)

to suffer from anemia, cytopenia and loss of weight often accompanied by ulceration of the gums and by diarrhea Monkeys on a diet devoid of the postulated vitamin M die in 26 to 100 days The vitamin is present in yeast and in liver and is therefore probably a member of the vitamin B complex Vitamin M deficiency causes a lowered microbic resistance in the gastrointestinal mucosa, and gingivitis and ulcerative colitis result from the action of pathogenic saprophytes (for example, *Bacterium dysenteriae* (Flesner), *Shiga bacillus*, etc ) normally present in the intestines There is a possibility[30] that a nutritional deficiency is also an essential etiological factor in human dysentery

## 11 Factor T

The existence of a fat soluble factor T has been postulated [31] The absence of this factor causes a decrease in the number of blood platelets (thrombocytosis) in rats and man Factor T is reported to occur in sesame oil and in egg yolks, but not in cod liver oil or olive oil The chemical nature of this compound is not known, but it has been observed that its activity is destroyed by ultraviolet irradiation

## 12 Factor U

Factor U is a growth promoting substance required by chicks [32, 33] It is present in yeast, wheat bran middlings alfalfa meal and to a lesser extent, in corn The factor is insoluble in ether acetone and isopropyl alcohol, but soluble in 50 per cent methanol and can be adsorbed on fuller's earth and charcoal The factor is stable in yeast when autoclaved for half an hour at pH 1 7 or 11 0 but is destroyed in alfalfa when autoclaved for five hours at 120° C

## 13 Folic Acid

Folic acid[34] (derived from the latin *folium*—leaf) occurs in green leaves of many kinds including grass Mushrooms and yeast are good sources It is also present in a number of animal tissues of which liver and kidney are the best sources

[30] G F McGinnes A L McLean F Spindle and K F Maxcy *Am J Hyg* 24 552 (1936) Editorial *J Am Med Assoc* 116 2169 (1941)

[31] E Schiff and C Hirschberger *Am J Diseases Children* 53 32 (1937) *Jahrb Kinderheilk* 146 [N F 96] 181 191 93 (1936) 147 [N F 97] 81 (1936)

[32] E L R Stokstad and P D V Manning *J Biol Chem* 125 687 (1938) *Science* 88 35 (1938)

[33] E L R Stokstad P D V Manning and R E Rogers *J Biol Chem* 132 463 (1940)

[34] H K Mitchell E E Snell and R J Williams *J Am Chem Soc* 63 2 84 (1941)

Folic acid has been isolated from spinach by adsorption on charcoal, precipitation with lead and silver salts and chromatographic adsorption on fuller's earth Folic acid has a molecular weight of about 500, contains nitrogen but no phosphorus or sulfur, and is acidic in character

Folic acid stimulates growth of rats and of various bacteria including *Streptococcus lactis* R , *Lactobacillus delbruckii* and *Lactobacillus casei* [35]

## 14 Growth Factor for *Lactobacillus casei*

The existence of a growth factor for *Lactobacillus casei* was demonstrated by the same method which was used for the isolation of folic acid (see page 525) The chemical characteristics of the growth factor are, however, different from those of folic acid This factor was isolated[36] from liver by adsorption on norite, followed by elution with ammonium hydroxide in methanol solution, followed by fractional precipitation with manganese salts The product obtained has the properties of a dinucleotide contain ing a purine and a pyrimidine nucleus Guanine was isolated from the hydrolysis products

## 15 Grass Juice Factor

The existence of a special factor necessary for optimal growth of rats[37] and of guinea pigs[38 39 40] has been postulated This factor is present in fresh grass[37] and in its juice[38] and has therefore been called grass juice factor Cereal grasses, rye grass, young white clover, peas, pea shells cabbage, turnip tops and spinach are excellent sources Young berries, cauliflower, canned peas and beans contain less Relatively little of this factor is present in apples celery, molasses peanuts turnips, lettuce and oats [41] Animal materials such as liver contain only small quantities The amount present in grass varies with the age of the plant, the maximum concentration being present in the growing plant while the mature and old plants contain considerably less [38 41] The active material can be preserved in forages by careful ensiling [4] Acid methods of ensiling are superior

35 E E Snell and W H Peterson *J Bact* 39 273 (1940)
36 E L R Stokstad *J Biol Chem* 139 475 (1941)
37 G O Kohler C A Elvehjem and E B Hart *J Nutrition* 14 131 (1937)
38 G O Kohler C A Elvehjem and E B Hart *Ibid* 15 445 (1938)
39 M D Cannon and G A Emerson *Ibid* 18 155 (1939)
40 G O Kohler S B Randle C A Elvehjem and E B Hart *Proc Soc Exptl Biol Med* 40 154 (1939)
41 S B Randle H A Sober and G O Kohler *J Nutrition* 20 459 (1940)
4 B C Johnson C A Elvehjem W H Peterson and H J Fagen *Ibid* 18 527 (1939)

to other methods especially to the molasses method and the dry method The factor is destroyed by heat and by oxidation [41]

The 'grass juice factor' appears to be different from all other known vitamins It can be precipitated from grass juice with acetone and then removed from this precipitate by means of acidified acetone When grass juice is deproteinized with chloroform and alcohol, the factor remains in the water phase and can be adsorbed on norite [43]

The grass juice factor is apparently readily absorbed by experimental animals and is secreted into milk [37, 42] Therefore the summer milk of cows contains considerable amounts of this factor while winter milk is practically devoid of it

In the absence of the grass juice factor rats and guinea pigs show a decline in weight and finally die The rat is less sensitive to a deficiency than the herbivorous guinea pig In addition to the loss in weight, some respiratory trouble was noted in most cases The lungs show inflammation and congestion and in isolated cases necrotic areas were observed [38]

## 16 Mouse Factor

Mice on a synthetic diet but not on stock diets, have been shown[44] to develop sore eyes, uni or bilateral, characterized by swelling and inflammation of the eyelids leading to closure of the eyes and blindness in many cases Simultaneously a dermatitis on the ventral aspect of the neck between the forelegs and extending almost up to the lower lip, and some loss of hair, especially on the face and neck occur Finally the animals die Rats on the other hand do not develop this disease when kept on the same diet The symptoms can be cured by normal diet Aqueous liver extracts and fresh flaked wheat germs are especially potent cures In many cases the symptoms could not be cured completely suggesting that the damage if too extensive is irreparable

## 17 Anti-Pernicious Anemia Factor

There is the possibility that the postulated extrinsic factor in the absence of which pernicious anemia occurs in man will eventually be found to be a vitamin Little is known about this factor In the presence of an intrinsic factor the extrinsic factor is supposedly converted into the compound which prevents the onset of pernicious anemia The latter com

[43] G O Kohler S B Randle and J R Wagner *J Biol Chem* 128 LV (1939)
[44] C. Carruthers *Science* 93 44 (1941)

pound is found predominantly in the liver, but the natural distribution of the extrinsic factor and its chemical characteristics are unknown This is due to the fact that it has been impossible to reproduce the human macro cytic hyperchromic anemia in animals

# APPENDIX

# THE VITAGENS

# THE VITAGENS

## ESSENTIAL FATTY ACIDS

### 1 Nomenclature and Survey

**Names**

Essential fatty acids
Vitamin F[1,2]
Vitamin $F_1$

**List of known essential fatty acids**

Linoleic acid $C_{18}H_{32}O_2$
Linolenic acid $C_{18}H_{30}O_2$
Arachidonic acid $C_{20}H_{32}O_2$

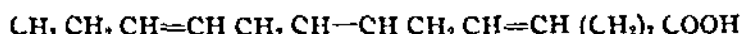
**Formulas**

Linoleic acid



$$CH_3\,(CH_2)_4\,CH{=}CH\,CH_2\,CH{=}CH\,(CH_2)_7\,COOH$$

Linolenic acid



$$CH_3\,CH_2\,CH{=}CH\,CH_2\,CH{-}CH\,CH_2\,CH{=}CH\,(CH_2)_7\,COOH$$

Arachidonic acid



$$CH_3\,CH{=}CH\,CH_2\,CH_2\,CH{=}CH\,CH_2\,CH_2\,CH{=}CH\,CH_2\,CH_2\,CH{=}CH\,(CH_2)_4\,COH$$

### 2 Chronology

1929 G O Burr and M M Burr[3] showed that the presence of highly unsaturated acids such as linoleic and linolenic acid in the diet is essential for normal growth of rats

1938 Turpeinen[4] found arachidonic acid more potent than any other of the investigated unsaturated acids and introduced the tentative hypothesis that the body needs this acid primarily Nunn and Smedley MacLean[5] established that arachidonic acid was deposited in the liver fat when linoleic acid was fed to rats

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[1] H M Evans S Lepkovsky and E A Murphy *J Biol Chem* 106 431 (1934)

[2] The Council on Pharmacy and Chemistry of the American Medical Association the American Society of Biological Chemists and the American Institute of Nutrition recommended that the term vitamin F should not be used in referring to linoleic or linolenic acids or any fatty acid or mixture of fatty acids *J Am Med Assoc* 113 589 (1939)

[3] G O Burr and M M Burr *J Biol Chem* 82 345 (1929) 86 618 (1930)

[4] O Turpeinen *J Nutrition* 15 351 (1938)

[5] L. C A. Nunn and I Smedley MacLean *Biochem J* 32 2178 (1938)

## 3 The Essential Fatty Acids as Vitagens

Much controversy has arisen over the question of whether or not the essential fatty acids should be classified as *vitamins*. In view of the definition of vitagens, as given on pages 5-6, the essential fatty acids are vitagens, since they are needed only in small quantities (0.1% of the food) and since they contribute to the mechanism of the transformation of energy, but are also building units of the phospholipides.

Which of the fatty acids are really essential is not known. The early work was done with linoleic and linolenic acids, both of which are able to counteract the deficiency symptoms, but apparently neither of these is stable in the organism. It has, however, been demonstrated[5] that after an intake of linoleic or linolenic acid, arachidonic acid was deposited in the liver fats. This may suggest that linoleic and linolenic acids are active precursors, whereas arachidonic acid is physiologically the most important compound. There are quite a number of other highly unsaturated fatty acids which occur naturally, but their vitagen nature has not been investigated as yet. In the following pages only the acids with known vitagen character will be discussed.

## 4 Occurrence

The essential fatty acids occur naturally both in plants and in animals. For a consideration of their occurrence, each fatty acid must be studied separately.

*1. Linoleic acid* occurs as a glyceride in most drying oils such as cotton seed, poppy seed, corn[6] and other oils. In animals, linoleic acid is present chiefly in the fatty acids of the phospholipides and to a lesser extent in the neutral fat.

*2. Linolenic acid*, like linoleic acid, occurs as a glyceride in most drying oils, such as linseed and perilla oils. Linolenic acid is usually absent from animal fats.

*3. Arachidonic acid* occurs predominantly in animal tissues. It is found mainly in the phospholipide fraction as a constituent of lecithin and cephalin,[7] but occurs also as a constituent of neutral fat. This acid has been isolated from heart[8] spleen,[8] liver[9] adrenal[8] and brain[10, 11] fatty acids.

[6] T. C. Taylor and J. M. Nelson *J. Am. Chem. Soc.* 42 1726 (1920)
[7] P. A. Levene and I. P. Rolf *J. Biol. Chem.* 51 507 (1922) 54 91 99 (1922)
[8] E. Klenk and O. von Schoenebeck *Z. physiol. Chem.* 209 112 (1932)
[9] P. Hartley *J. Physiol.* 38 353 (1909)
[10] E. Klenk and J. Dittmar *Z. physiol. Chem.* 244 203 (1936)
[11] D. Wesson *J. Biol. Chem.* 60 183 (1924)

## 5. Isolation

The isolation of the essential fatty acids comprises the isolation of the total fatty acids from plant or animal materials followed by the separation of the individual fatty acids.

Plant material is either saponified as such or the oil present in plants, such as in seeds, is pressed out first, and then saponified. Alkali or alkaline earth hydroxide is usually used for these saponifications. The acids are usually extracted from the saponification mixture with an organic solvent. Linoleic and linolenic acids are separated from the other fatty acids by the addition of bromine whereby the tetra- and the hexabromide, respectively, precipitate. The brominated acids are separated and debrominated by means of zinc and alcohol. Thus a mixture of linoleic and linolenic acids is obtained which can be separated by means of the different solubilities of their zinc salts in alcohol.

Animal materials are either saponified as such or the total fat is extracted or separated into the phospholipides and true fats. The latter procedure is carried out by dehydrating the material either by a drying process or better by means of acetone. The total fatty material is then extracted with alcohol preferably at elevated temperature and in the presence of inert gases such as nitrogen. After removal of the solvent, the phospholipide fraction is precipitated with acetone while the true fats go into solution. This division into neutral fats and phospholipides is not a quantitative procedure, but serves for a gross separation. The fractions are then saponified and worked up as described for plant material. Upon bromination, the octabromide of arachidonic acid is obtained which yields arachidonic acid upon debromination.

The yields of the unsaturated acids obtained by the bromination-debromination procedure are rather low, but the purity is considered to be high, at least in the case of linoleic and linolenic acids. To a certain extent, however, these acids and especially arachidonic acid undergo a rearrangement of the position of the double bonds.

A principally different method can be used for the isolation of the unsaturated fatty acids, namely, the direct isolation by fractional crystallization at low temperature. The yields by this method are high but the purity of the product is not as satisfactory. Practically, the phosphatide fraction is used for the isolation and the phosphatides are converted into methyl esters by direct alcoholysis.[12] Fractional crystallization is then carried out at various low temperatures, for example, consecutively at −20°,

[12] G. Y. Shinowara and J. B. Brown, *Oil & Soap* 15, 151 (1938).

−40° and −80° C By this method linoleic acid has been obtained from cottonseed and from corn oils in 90–94% purity,[13] linolenic acid from linseed and from perilla oils in 85–88% purity[14] and arachidonic acid from adrenal phosphatides in 70–75% purity [15] Further purification, for example, of the methyl arachidonate, can be effected by fractional distillation, whereby a product of 95% purity is obtained [15]

## 6 Properties

All essential fatty acids have similar solubility characteristics They are insoluble in water, but soluble in alkali and in organic solvents

(1) Linoleic acid Colorless liquid at room temperature M p 11° C
(2) Linolenic acid Colorless liquid B p 230–232° C /17 mm Hg
(3) Arachidonic acid Colorless crystals M p 77 C

## 7 Chemical Constitution

All essential fatty acids are carboxylic acids as evident from the formation of various salts, analytical data and the formation of various esters

*1 Linoleic acid* has the empirical formula $C_{18}H_{32}O_2$ and contains two double bonds as evident from the formation of a tetrabromide and of tetrahydroxy stearic acid (sativinic acid) upon mild oxidation with permanganate [16] Other oxidation products are azelaic acid, oxalic acid and *n* caproic acid [17] Upon reduction with hydriodic acid and phosphorus, stearic acid is obtained [18] Linoleic acid is therefore, Δ 9, 12 octadecadienoic acid

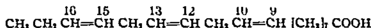

$$CH_3(CH_2)_4CH{=}CH\,CH_2\,CH{=}CH(CH_2)_7COOH$$

*2 Linolenic acid* has the empirical formula $C_{18}H_{30}O_2$ and contains three double bonds since upon addition of bromine a hexabromo derivative is formed Upon reduction stearic acid is obtained Linolenic acid is therefore a straight chain fatty acid [19] Oxidation with ozone yields propionaldehyde, malonic aldehyde carboxylic acid and azelaic aldehyde

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[13] J B Brown and G C Stoner *J Am Chem Soc* 59 3 (1937) J B Brown and J Frankel *Ibid* 60 54 (1938)
[14] G Y Shinowara and J B Brown *Ibid* 60 2734 (1938)
[15] G Y Shinowara and J B Brown *J Biol Chem* 134 331 (1940)
[16] A Rollett *Z physiol Chem* 62 410 (1909)
[17] G L Goldsobel *Chem Ztg* 30 825 (1906)
[18] K Peters *Monatsh* 7 552 (1886)
[19] F Erdmann and F Bedford *Ber* 42, 1324 (1909)

carboxylic acid The three double bonds of linolenic acid are therefore in Δ 9 12 15 positions according to the formula



$$\overset{16}{CH_3}\,CH_2\,\overset{15}{CH}{=}\overset{13}{CH}\,CH_2\,\overset{12}{CH}{=}\overset{10}{CH}\,CH_2\,\overset{9}{CH}{=}CH\,[CH_2]_7\,COOH$$

*3 Arachidonic acid* has the empirical formula $C_{20}H_{32}O_2$ and contains four double bonds An octabromo arachidonic acid is formed upon bromination The straight chain structure is established by total hydrogenation and comparison with a synthetic *n* eicosanic acid [20] The position of the four double bonds has been established by ozonolysis which yielded caproic, acetic succinic, malonic and glutaric acids, acetaldehyde and carbon dioxide Arachidonic acid therefore has been assigned the structure of Δ 5 8 11, 14 eicosa tetrenoic acid [20a]



$$CH_3\,[CH_2]_3\,[CH{=}CH\,CH_2]_4\,CH_2\,CH_2\,COOH$$

An eicosa tetrenoic acid which is apparently different from arachidonic acid, has been isolated from sardine oils This acid has the double bonds in 4, 8 12 and 16 positions since upon oxidation succinic and butyric acids exclusively are obtained in almost quantitative yields [21] The biological efficacy of this acid has not been investigated as yet

## 8 Synthesis

None of the essential fatty acids has been synthesized

## 9 Biogenesis

The origin and the biogenesis of linoleic acid and of linolenic acid in plant material are largely unknown It has been speculated that certain plants may contain a dehydrogenation mechanism whereby saturated fatty acids are converted into unsaturated fatty acids However it is just as probable that the unsaturated fatty acids are obtained by total synthesis as is the case of the saturated fatty acids

A better insight exists concerning the biogenesis of arachidonic acid Upon feeding of linoleic or linolenic acid to rats arachidonic acid is deposited in the tissues [22] This proves a partial synthesis of this acid

[20] G Y Shinowara and J B Brown *J Biol Chem* 134 331 (1940)

[20a] D E Dolby J C A Nunn and I Smedley MacLean *Biochem J* 34 1422 (1940) D T Mowry W B Brode and J B Brown *J Biol Chem* 142 679 (1942) C J Arcus and I Smedley MacLean *Biochem J* 37 1 (1943)

[21] Y Toyama and T Tsuchiya *Bull Chem Soc Japan* 10 296 (1935)

[22] L C A Nunn and I Smedley MacLean *Biochem J* 32 2178 (1938)

in the organism of the rat and indicates that rats are incapable of synthesizing a precursor of arachidonic acid

## 10 Specificity

It has already been mentioned that of all the fatty acids investigated, only linoleic, linolenic and arachidonic acids show activity Besides the free acids, the esters are active The esters of the lower aliphatic alcohols have been demonstrated to be active and it is expected that all esters which can be hydrolyzed in the organism will prove effective

The relative efficacy of the active acids, tested as their methanol esters, is recorded in the following table showing the symptom efficacy according to the skin test and growth test on a comparative basis arbitrarily designating the activity of linoleic acid in both tests as 1

| Acid | Efficacy in skin test | Efficacy in growth test |
|---|---|---|
| Arachidonic acid[23] | 1 | 6 |
| Linoleic acid | 1 | 1 |
| Linolenic acid | 1/8 | 1/2 |
| Docosa hexaenoic acid ($C_{22}H_{32}O_2$)[24] | 0 | Little |
| Linusic and isolinusic acid | 0 | 0 |

Tetrahydroxy stearic acids, dioxido stearic acid, 9,10,12 trihydroxy stearic acid and chaulmoogric acid are inactive

## 11 Determination

### (a) Chemical Methods

The only known chemical method for the determination of the essential fatty acids is the precipitation of their bromine addition compounds which has been described on page 533

A number of color reactions have been proposed[2] for the determination of the essential fatty acids, which are based upon reactions of the double bonds Thus the transfer of hydrogen from aromatic amines such as *p* phenylene diamine either alone or in the presence of phenols, for example, *o* cresol, to the essential fatty acids has been studied The colors developed depend upon the concentration used and the time Initially a violet then

[23] E M Hume L C A Nunn I Smedley MacLean and H H Smith *Biochem J* 34 879 (1940)
[24] E H Farmer and F A Van de Heuvel *J Chem Soc* 1938 427
[25] G Woker and P Bernhard *Helv Chim Acta* 24 98 (1941)

an olive green and finally a gray color are observed This reaction can be made considerably more sensitive by the addition of hydrogen peroxide Another reaction which has been studied, is the addition of iodine to the double bonds All these reactions are however, not specific for the essential fatty acids since they are given by other unsaturated but non essential fatty acids by carotenoids, etc

### (b) *Biological Methods*

(a) **The Rat Growth Method**[26] This method led to the discovery of the essential fatty acids and can be used either prophylactically or as a curative method and is based on increase in weight following the feeding of the essential fatty acids to young rats which have been depleted of these acids The depletion of this factor is a very slow process and may take many months

(b) **The Rat Skin Test**[27] In this curative method, the criterion is the healing of the characteristic dryness and scurfiness on the dorsal surface of the hind feet and front of the ankles Instead of feeding the essential fatty acid preparation, the material may also be applied topically to the skin[28]

(c) **The Oestrus Cycle Test**[29] Irregular ovulation observed during essential fatty acid deficiency, has been used for the determination of the deficiency of these factors and the restoration of the oestrus cycle toward normal has been used for the determination of the presence of the essential fatty acids

## 12 Standards

There is no recognized standard fatty acid for comparison of its biological effect with that of unknown material Since it is desirable to set up such a standard reference essential fatty acid it is recommended that arachidonic acid be adopted since all available evidence indicates that this is the acid which is produced in the organism from the other known essential acids

## 13 Physiology

Little is known about the physiology and the mode of action of the essential fatty acids Practically all experimental work has been carried out with rats

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[26] G O Burr and M M Burr *J Biol Chem* 82 345 (19?9) 86 618 (1930)
[27] E M Hume L C A Nunn I Smedley MacLean and H H Smith *Biochem J* 32 ?16? (1938)
[28] M I Shepherd and D R Linn *Drug Cosmetic Ind* 38 6?9 (1936)
[29] O Turpeinen *J Nutrition* 15 351 (1938)

Linoleic and linolenic acids and their simple esters are absorbed from the intestinal tract Linolenic acid is metabolized directly after entering the organism and is not stored as such unless it is administered in excessive doses[30] Linoleic acid, on the other hand, has been found invariably in all investigated animals and in man in the fatty acids of the phospholipides and to a lesser extent in the neutral fats The main unsaturated acid in the phospholipides is arachidonic acid The essential fatty acids are held in the phospholipides with extreme tenacity[31] and it takes therefore several months before a rat is completely depleted of these acids

It is possible that linoleic and linolenic acids are really precursors of the active form Linolenic acid cannot be demonstrated to be present in the organism although its biological efficacy is established Linoleic and linolenic acids are converted in the organism into arachidonic acid[32, 33] which is the most active acid known Besides arachidonic acid, other highly unsaturated fatty acids such as an acid $C_{20}H_{34}O_2$, dihydro arachidonic acid, docosa penta enoic acid, etc , are synthesized in the body from linoleic and linolenic acids Whether or not some of these act as "essential fatty acids" is not known

The mechanism of the action of the essential fatty acids is not quite clear Apparently these acids act in phosphatides and take part in the mechanism of utilizing fats It has been suggested[34] that the first action is to load up the connective tissue cells of the fat depots with fats If only small amounts of the essential fatty acids are administered this effect can be demonstrated separately After the depots are filled up with fat and additional amounts of the essential fatty acids are offered, growth takes place Simultaneously the excessive fat deposits disappear

The essential fatty acids are, however, not only connected with the movement of fats but also with the utilization of fats It has been shown in *in vitro* experiments that unsaturated fatty acids catalyze the oxidation of saturated fatty acids without being affected themselves

From experiments with fat starved rats, the conclusion was drawn[35] that the essential fatty acids are necessary for the formation of new tissue but not for the maintenance of the normal metabolism of the cell

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[30] N R Ellis and H S Isbell *J Biol Chem* 69 219 (1926) E Klenk *Z physiol Chem* 206 25 (1932) R H Snider and W R Bloor *J Biol Chem* 99 555 (1933)

[31] R G Sinclair *Proc Soc Exptl Biol Med* 27 1059 (1930)

[32] N R Ellis and H S Isbell *J Biol Chem* 69 219 (1926) H C Eckstein *Ibid* 81 613 (1929) J M Spadola and N R Ellis *Ibid* 113 205 (1936)

[33] L C A Nunn and I Smedley MacLean *Biochem J* 32 2178 (1938)

[34] I Smedley MacLean and L. C. A. Nunn *Ibid* 34 884 (1940)

[35] I Smedley MacLean and E M Hume *Ibid* 35 990 (1941)

## 14 Deficiency Syndrome

A deficiency of the essential fatty acids causes a typical dermatosis in rats characterized by general dryness of the skin and thinness with much scurf especially on both the fore and hind paws and on the ears and tail The latter may appear to be annulated It should be noted that there is no edema present as is in the otherwise quite similar symptoms caused by a vitamin $B_6$ deficiency In fat deficiency the lacrimal glands are also affected and irregular ovulation and lesions of the kidney and of the urinary tract occur Abnormal reproduction and deficient lactation have been observed In young rats cessation of increase in length and weight occurs Sexual development is also delayed

Clinical symptoms of a deficiency of the essential fatty acids in man are not known There are, however, some observations which indicate that in certain eczema, and especially in infantile eczema, favorable clinical results can be obtained when oils containing unsaturated fatty acids are administered [36] In such pathological cases it was found that the fatty acids from the blood serum contained a significantly lower concentration of unsaturated compounds than those of healthy individuals By the time a clinical improvement was noticed, the concentration of unsaturated fatty acids had risen to essentially normal levels

### (a) *Clinical Test Methods*

There are no accurate methods for determining a deficiency of the essential fatty acids It has been proposed to assay the actual concentration of unsaturated fatty acids in the serum since it was found that in rats,[37] goats[38] and dogs[39] the degree of unsaturation diminished with a lowered intake of the unsaturated fatty acids These methods however, have not been perfected and do not permit a differentiation of the essential fatty acids from non essential, unsaturated fatty acids

## 15 Requirements

The requirements of the essential fatty acids are largely unknown In rats, a daily intake of about 14 mg methyl arachidonate produces optimum effects [40] but decreased amounts show less favorable responses

[36] A E Hansen *Proc Soc Exptl Biol Med* 30 1198 (1933) 31 160 161 (1933) T Cornbleet *Arch Dermatol Syphilol* 31 224 (1934)
[37] A E Hansen and G O Burr *Proc Soc Exptl Biol Med* 30 1 00 1201 (1933)
[38] H H Williams and L A Maynard *J Dairy Sci* 17 223 (1934)
[39] A. E Hansen W R Wilson and H H Williams *J Biol Chem* 114 209 (1936)
[40] E M Hume L C. A. Nunn I Smedley MacLean and H H Smith *Biochem J* 34 879 (1940)

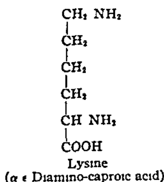
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## ESSENTIAL AMINO-ACIDS

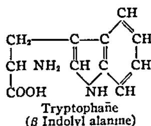
Among the amino acids which are normal constituents of dietary pro teins, ten are essential Those amino acids are defined[1 2] as essential" which must be present in the diet of young rats, in addition to all other energy and building unit requisites and the vitamins, to insure optimal growth That is, these amino acids cannot be synthesized in the animal organism or at least not at a rate necessary for normal growth These ten amino acids are [1 2] lysine, tryptophane, histidine, phenyl alanine, leu cine, isoleucine, threonine, methionine, valine, arginine

A deficiency of any one or of all of these ten amino acids causes an inhibitory effect upon growth The same amino acids which are essential for rats are also essential for dogs [3] It is reasonable to assume that they are also indispensable for the human body, but no definite information regarding the special amino acid requirements of man is available

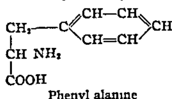
Arginine holds a somewhat unique position Rats on an arginine free diet are able to synthesize some of their requirements since the animals show a



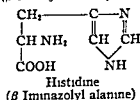
Lysine
($\alpha$ $\epsilon$ Diamino-caproic acid)



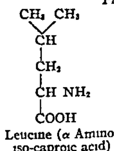
Tryptophane
($\beta$ Indolyl alanine)



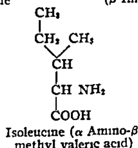
Phenyl alanine



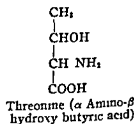
Histidine
($\beta$ Iminazolyl alanine)



Leucine ($\alpha$ Amino iso-caproic acid)

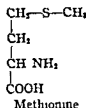


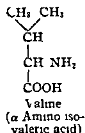
Isoleucine ($\alpha$ Amino-$\beta$ methyl valeric acid)

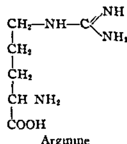


Threonine ($\alpha$ Amino-$\beta$ hydroxy butyric acid)

[1] W C Rose *Physiol Rev* 18 109 (1938) [2] W C Rose *Science* 86 298 (1937)
[3] W C Rose and E E Rice *Ibid* 90 186 (1939)


Methionine


Valine
($\alpha$ Amino iso-
valeric acid)


Arginine

growth of 70–80% in comparison with animals which received arginine [4] In mice the synthesis of arginine from ornithine has been demonstrated by means of labeling the compounds with deuterium [5] Adult dogs can get along without an external supply of this amino acid Chicks, on the other hand, must rely upon dietary arginine[6] since this amino acid is needed as a structural building unit of creatine for feather formation

While retarded growth is a somewhat non specific symptom for a vitamin or vitagen deficiency specific deficiency symptoms are known for a few of the indispensable amino acids The fright disease of dogs has for example, been correlated with inadequate amino acid supply [7] A deficiency of lysine causes anemia in rats [8] Cataract and other ocular changes have been observed in young rats on a tryptophane deficient diet [9] Rats deprived of valine show a disorder of the nervous system characterized by sensitivity to touch and severe lack of coordination in movement [10] Young chicks fed a diet devoid of arginine and glycine develop a typical paralysis [6] For the effects caused by a deficiency in methionine see pages 543 and 549 Other specific deficiency syndromes will undoubtedly be observed upon further studies

The exact requirements of the essential amino acids for man and various animal species are not known The minimum amount necessary to support normal growth has been determined only for rats [10] The total amount of essential amino acids corresponds to 5 8% of the food consumed with the following figures for the individual amino acids lysine 1 0% leucine 0 9% phenyl alanine 0 7% valine 0 7% threonine 0 6% methionine 0 6%, isoleucine 0 5% histidine 0 4% tryptophane 0 2% and arginine 0 2%

[4] C W Scull and W C Rose *J Biol Chem* 89 109 (1930)
[5] R F Clutton R Schoenheimer and D Rittenberg *Ibid* 132 227 (1940)
[6] A Arnold C L Kline C A Elvehjem and E B Hart *Ibid* 116 699 (1936) D M Hegsted G M Briggs C A Elvehjem and E B Hart *Ibid* 140 191 (1941)
[7] A Arnold and C A Elvehjem *J Am Vet Med Assoc* 95 303 (1939)
[8] A G Hogan E L Powell and R E Guerrant *J Biol Chem* 137 41 (1941)
[9] J R Totter and P L Day *Ibid* 140 CXXXIV (1941)
[10] W C Rose *Science* 86 298 (1937)

## ESSENTIAL CARBOHYDRATES

In analogy to the discovery that besides the energy bearing and building unit supplying compounds of the class of fats and proteins there are some fats and proteins which are also protective food constituents and which are therefore classified as vitagens, there may be specific carbohydrates which are vitagens Ascorbic acid and inositol are examples of essential carbo hydrates which are vitamins Whether or not other carbohydrates exist which are essential food constituents is not known If carbohydrates as food constituents would be utilized only to supply energy, it should be pos sible to replace all carbohydrates with the exception of the carbohydrates of vitamin character by some other energy bearing foods such as fats or proteins This is, however, not the case The animal organism main tains, for example, in the blood, a certain normal glucose concentration and it has been shown that the organism is able to synthesize a certain amount of glucose for this purpose [1,2,3] Animals on a completely carbohydrate free diet, however, do not survive, apparently because the amount of glu cose synthesized is not sufficient to maintain the necessary carbohydrate concentration in the organism It is thus possible that either carbohydrates in general or some specific carbohydrates may, eventually, be found to be essential protective food constituents

There is some evidence for the existence of an additional growth factor of the carbohydrate class at least for the growing chick This growth fac tor was first encountered in rice and has therefore been called ' rice factor " This factor is apparently not present in yeast [4] It has also been found in cartilage and the effect has been shown to be caused by the glucuronic acid component of chondroitin [5] The effect does not appear to be very specific but is also brought about by a number of other carbohydrates, such as gum arabic, sodium alginate gluconic acid galactonic lactone, arabinose and xylose, while sugars like *d* ribose and rhamnose are apparently inac tive [6]

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[1] R D Cramer and G B Kistiakowsky *J Biol Chem* 137 549 (1941)

[2] J B Conant R D Cramer A B Hastings F W Klemperer A K Solomon and B Vennesland *Ibid* 137 557 (1941)

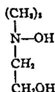
[3] A. K Solomon B Vennesland F W Klemperer J M Buchanan and A B Hastings *Ibid* 140 171 (1941)

[4] D W Hegsted J J Oleson C A Elvehjem and F B Hart *Poultry Sci* 19 167 (1940) F L R Stokstad P D V Manning and R F Rogers *Ibid* 19 107 (1940)

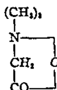
[5] H J Almquist E L R Stokstad E Mecchi and P D V Manning *J Biol Chem* 134 213 (1940) H J Almquist E Mecchi E L R Stokstad and P D V Manning *Ibid* 134 465 (1940)

[6] E L R Stokstad H J Almquist E Mecchi P D V Manning and R L Rogers *Ibid* 137 373 (1941)

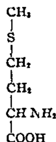
# CHOLINE AND RELATED COMPOUNDS AND THE ESSENTIAL, TRANSFERABLE METHYL GROUP



Choline



Betaine



Methionine

## 1 Chronology

1894 HOFMEISTER[1] postulated the transfer of methyl groups in metabolism as the result of *in vivo* formation of methyl selenides and tellurides

1917 THOMPSON[2] concluded that the synthesis of creatine in the animal organism is the result of methyl transfer

1932 BEST and co-workers[3] showed that in rats the addition of choline or betaine to a diet high in fat prevents the deposition of excess amounts of fats in the liver

1935 BEST and co workers[4] presented evidence that choline is an accessory food factor for rats

1937 TUCKER and ECKSTEIN[5] found that methionine exerted an effect on liver fat similar to that of choline

1939 DU VIGNEAUD and co workers[6] observed that the essential amino acid methionine can be replaced in the diet of rats by homocysteine when choline or betaine is given simultaneously and suggested that this effect is due to the anabolic formation of methionine by methyl transfer

1940 BORSOOK and DUBNOFF[7] and DU VIGNEAUD and co workers[8] proved that methyl transfer occurred in the synthesis of creatine in the animal organism DU VIGNEAUD[8] expressed the view that the transferable methyl group may be an essential dietary constituent

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[1] F Hofmeister *Arch expll Path Pharmakol* 33 198 (1894)

[2] Thompson *J Physiol* 51 347 (1917)

[3] C H Best J M Hershey and M E Huntsman *Am J Physiol* 101 7 (1932) *J Physiol* 75 56 (1932) C H Best and M E Huntsman *Ibid* 75 405 (1932)

[4] C H Best M E Huntsman E W McHenry and J H Ridout *J Physiol* 84 38P (1935)

[5] H F Tucker and H C Eckstein *J Biol Chem* 121 479 (1932) 126 117 (1938)

[6] V du Vigneaud J P Chandler A W Moyer and D M Keppel *Ibid* 131 57 (1939)

[7] H Borsook and J W Dubnoff *Ibid* 132 559 (1940)

[8] V du Vigneaud J P Chandler M Cohn and G B Brown *Ibid* 134 787 (1940)

## 2 The Active Compounds and Their Properties

The number of naturally occurring compounds which may serve, among other purposes, as suppliers of the essential transferable methyl group is not known However, besides choline, methionine and betaine are known to act in this capacity, especially in the absence of the former

**Choline** Choline is a colorless viscid strongly alkaline liquid and is very hygroscopic It absorbs carbon dioxide from the air and easily forms salts—for example the chloride borate picrate etc Choline is very soluble in water and alcohol but is insoluble in ether The salts are white hygroscopic crystals which are soluble in water and in alcohol Their aqueous solutions are practically neutral

**Methionine** M p 283° C under decomposition (Darkening at 278° C) $[\alpha]_D^{25} = -6.87°$ C in water

**Betaine** Betaine forms very hygroscopic crystals which lose their water at 100° C Betaine melts at 293 C under rearrangement into the methyl ester of dimethyl amino acetic acid

## 3 Pathological States Caused by a Deficiency of the Active Compounds

A deficiency of choline, betaine or methionine in the diet of young rats or dogs causes the deposition in the liver of fat[9] and to a lesser extent also of cholesterol esters [10] These are removed or their deposition prevented when choline or one of the other mentioned compounds is fed These substances are called lipotropic factors At the same time a diffuse nodular cirrhosis of the liver occurs and has been observed in rats,[11, 12] dogs [13] rabbits [14, 15] and guinea pigs [15] The deficiency of these compounds furthermore causes hemorrhagic degeneration of the kidneys characterized by symmetrical hemorrhagic necrosis of the cortex [16, 17, 18, 19] An involution of the thymus gland, an enlargement of the spleen a transformation of the

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[9] C H Best J M Hershey and M E Huntsman *Am J Physiol* 101 7 (1932) *J Physiol* 75 56 (1932) C H Best and M E Huntsman *Ibid* 75 405 (1932)

[10] C H Best and J H Ridout *J Physiol* 78 415 (1933) 84 7P (1935) A V Stresser I Mc Quarrie and J A Anderson *Proc Soc Exptl Biol Med* 33 595 (1936) H P Himsworth *Acta Med Scand* Supplement 90 158 (1938)

[11] H Blumberg *U S Pub Health Service Pub Health Repts* 55 534 (1940) H Blumberg and H G Grady *Proc Am Soc Biol Chem* April 1941 H Blumberg and E V McCollum *Science* 93, 598 (1941) R D Lillie F S Daft and W H Sebrell *Pub Health Rep* 56 1255 (1941) F S Daft W H Sebrell and R D Lillie *Proc Soc Exptl Biol Med* 48 228 (1941)

[12] P György and H Goldblatt *Proc Soc Exptl Biol Med* 46 492 (1941) P György E C Poling and H Goldblatt *Ibid* 47 41 (1941)

[13] I L Chaikoff and C L Connor *Ibid* 43 638 (1940)

[14] A R Rich and J D Hamilton *Bull Johns Hopkins Hosp* 66 185 (1940)

[15] M A Spellberg and R W Keeton *Am J Med Sci* 200 688 (1940)

[16] W H Griffith and N J Wade *J Biol Chem* 131 567 (1939) 132 677 (1940)

[17] W H Griffith *J Nutrition* 21 291 (1941) W H Griffith and D J Mulford *J Am Chem Soc* 63 929 (1941)

[18] P György and R E Eckhardt *Biochem J* 34 1143 (1940)

[19] L Rane and Y Subbarow *J Biol Chem* 134 455 (1940)

lymph nodes to hemolymph nodes, and in more severe cases hemorrhages in the eye have been observed [20, 21] These compounds are also necessary for normal growth and lactation of the rat In the nursing young rat a flaccid paralysis of the hind leg occurs [19, 22] In albino rats a rustiness of the fur has been observed as the result of a diet low in choline [23]

In chicks choline deficiency causes decreased or discontinued egg production, increased mortality and abortion of egg yolks [24] In turkeys and in chicks choline deficiency is characterized by perosis and by slow growth [25, 26, 27]

Choline deficiency is also manifested by high non protein nitrogen in the blood [28] Prolonged deficiency causes a loss of the ability of the liver to store glycogen and to excrete dye [29]

## 4 Specificity Studies

The naturally occurring suppliers of the transferable methyl group show a relatively high specificity Many proteins are active due to their content of methionine or due to the fact that betaine may be formed from some of the amino acids in the protein [30] However the methyl groups of all compounds, which have methyl groups attached to quaternary nitrogen or sulfur are not transferable In order to be available for transmethylation, the methyl group must be bound on specific molecules Thus in choline the hydroxyl group must be free or in a readily available form since the ethers are inactive [31] Choline salts such as choline chloride and betaine aldehyde are active [31] On the other hand, the nitrogen of choline may be replaced by phosphorus[31] without loss of activity The methyl groups of creatine,[32] S methyl cysteine[33, 34] or of the betaines from threonine, serine or allothreonine[35] are not transferable

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[20] K Christensen *Proc Am Soc Biol Che* 1940 XX
[21] R W Engel and W D Salmon *J Nutrition* 22 109 (1941)
[22] B Sure *Ibid* 19 71 (1940)
[23] H S Owens M Trautman and F Woods *Science* 93 406 (1941)
[24] O D Abbott and C U DeMasters *J Nutrition* 19 47 (1940)
[25] T H Jukes *J Biol Chem* 134 789 (1940) *J Nutrition* 20 445 (1940)
[26] D M Hegsted R C Mills C A Elvehjem and F B Hart *J Biol Chem* 138 459 (1941)
[27] T H Jukes *Proc Soc Exptl Biol Med* 46 155 (1941)
[28] W H Griffith and D J Mulford *J Nutrition* 21 633 (1941) R W Engel and W D Salmon *Ibid* 22 109 (1941)
[29] D L MacLean J H Ridout and C H Best *Brit J Exptl Path* 18 345 (1937)
[30] A. W Beeston H J Channon J V Loach and H Wilkinson *Biochem J* 30 1040 (1936) C H Best R Grant and J H Ridout *J Physiol* 86 337 (1936)
[31] A D Welch and M S Welch *Proc Soc Exptl Biol Med* 39 7 (1938)
[32] V du Vigneaud J P Chandler M Cohn and G B Brown *J Biol Chem* 134 787 (1940)
[33] H J Channon M C Manifold and A P Platt *Biochem J* 34 866 (1940)
[34] A D Welch *J Biol Chem* 137 173 (1940)
[35] H E Carter and D B Melville *Ibid* 133 109 (1940)

Choline derivatives which contain alkyl groups other than the methyl group and even the methyl diethyl homolog[34] are unable to support growth of rats on a diet containing homocysteine,[36] but are potent lipotropic agents[37] and prevent the occurrence of hemorrhagic kidneys.[34] Arsenocholine, which is active as a lipotropic factor[31, 38] and prevents the hemorrhagic kidney condition in rats and perosis in turkeys,[34] does not transfer the methyl group to homocysteine.[34]

## 5 The Physiological Action of the Active Compounds

According to the tentative classification of choline as a vitagen, this substance is utilized in the animal organism as a building unit and is at the same time concerned with the regulation of metabolic processes. Choline serves as a building unit in many phosphatides, for example, in lecithins and in sphingomyelins. Choline is present in the phospholipides of practically all cells. In addition, choline regulates the turnover of fat[39] and of phospholipides,[40] for example, in the liver, and has for that reason often been classified as a vitamin.[41] In the absence of choline, fat is deposited in the liver, but this deposition is removed when choline becomes available. A dietary deficiency of choline becomes apparent from disturbances of the fat metabolism in spite of the presence in the body of very large amounts of choline containing phospholipides.[4] In addition to these functions, choline is utilized in the animal organism after acetylation as a blood pressure lowering substance. The process of producing acetylcholine is intimately connected with nerve impulses and special enzyme systems are readily available in the organism to acetylate choline when needed and to hydrolyze the active acetyl compound to the free choline which is relatively incapable of influencing the blood pressure.

Choline can be synthesized to a limited extent in the animal organism. This is apparent, for example, from the fact that deuterium containing choline can be isolated after feeding methionine, the sulfur bound methyl group of which contained deuterium.[43] Choline synthesis is furthermore indicated by the fact that the choline content of rats on choline-free diets

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36 V du Vigneaud, J P Chandler, A W Moyer and D M Keppel, *J Biol Chem* 131, 57 (1939)

37 H J Channon and J A B Smith, *Biochem J* 30, 110 (1936); H J Channon, A P Platt and J A B Smith, *Ibid* 31, 1736 (1936)

38 A D Welch, *Proc Soc Exptl Biol Med* 35, 107 (1936); C H Best and J H Ridout, *Can Med Assoc J* 39, 188 (1938)

39 See the review by C H Best and J H Ridout, *Ann Rev Biochem* 8, 349 (1939)

40 I Perlman and I L Chaikoff, *J Biol Chem* 127, 211 (1938); 128, 735 (1939); 130, 593 (1339)

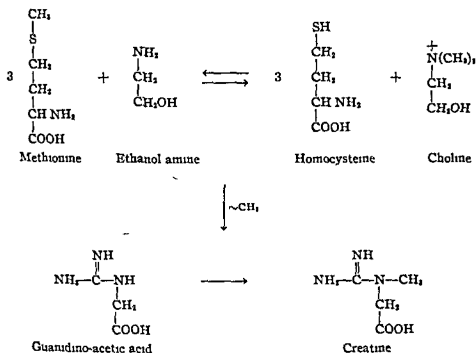
41 P György and H Goldblatt, *J Exptl Med* 72, 1 (1940); C G King, *Ann Rev Biochem* 8, 371 (1939); H C Sherman, *Chemistry of Food and Nutrition*, New York 1941, p 395

42 F X Aylward, H J Channon and H Wilkinson, *Biochem J* 29, 169 (1935)

43 V du Vigneaud, J P Chandler, M Cohn and G B Brown, *J Biol Chem* 134, 787 (1940)

increased with the weight of the animals [44] Preformed choline must, however be supplied to the animal for optimal physiological performance

The methyl groups attached to the quaternary nitrogen are partly responsible for the physiological action of choline They serve as methylating agents in anabolic processes for example in the synthesis of creatine from guanidino acetic acid Choline can be replaced in this reaction by other compounds with transferable methyl groups ($\sim CH_3$) such as methionine and betaine Another physiologically important methylation is the synthesis of methionine from homocysteine and choline On the other hand choline can be built up from methionine and ethanol amine This series of reactions is shown in the following scheme




The action of choline however, cannot be due solely to the available methyl groups For example, the ethyl derivatives of choline cannot transfer methyl groups since the compounds are devoid of them and either do not transfer the ethyl group or the ethyl group is inactive In any event, these compounds are unable to support growth of rats on a choline and methionine free diet in the presence of homocysteine [45] Arsenocholine

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[44] H P Jacobi C A Baumann and W J Meek *J Biol Chem* 138 571 (1941)

[45] V du Vigneaud J P Chandler A W Moyer and D M Keppel *Ibid* 131 57 (1939)

also is unable to transfer methyl groups [46] However, these compounds are active as lipotropic factors, and they prevent the hemorrhagic kidney condition and the perosis encountered in animals kept on a diet devoid of choline

## 6 Requirements

Choline (methionine or betaine) is required by all investigated animals, such as the rat, dog rabbit, guinea pig, chick turkey, etc Choline is also an essential growth factor for bacteria, for example, for the *Pneumococcus bacillus* [47]

Generally the young, growing organism needs more choline than the adult organism During lactation the requirements are increased Rats need 10–20 mg choline per day to prevent the deposition of excess amounts of fats in the liver and to maintain the fat deposition at a normal level [48] The choline requirement of dogs is about 35 mg per kilogram of body weight [49] Chicks need about 75 mg daily [50]

In the absence of choline, the animal organism is able to utilize effectively methionine or betaine The efficacy of these compounds is, however somewhat lower than that of choline (estimated efficacy 30%)

[46] A D Welch *Ibid* 137 173 (1940)
[47] L Rane and Y Subbarow *Ibid* 134 455 (1940)
[48] H J Channon J V Loach and G R Tristram *Biochem J* 32 13?? (1938)
[49] C Entenman and I I Chaikoff *J Biol Chem* 138 477 (1941)
[50] O D Abbott and C U DeMasters *J Nutrition* 19 47 (1940)

## ESSENTIAL ORGANIC SULFUR-CONTAINING COMPOUNDS

There is the possibility that organic chemical compounds containing sulfur belong to the class of protective foods[1] and should be classified as vitagens Sulfur containing organic compounds act not only as structural building units for example in hair and nails, but are also of specific and functional importance for growth and maintenance of life A deficiency of sulfhydryl compounds brings about death Sulfhydryl compounds apparently act as activators (and inhibitors) of a number of enzyme systems probably by virtue of their ability to undergo reversible oxidation to disulfide compounds and reduction to the original sulfhydryl compounds They act, for example, as activators in the aerobic and anaerobic fermentation and in the oxidation of glucose in propionic acid bacteria [3] In experiments with the *Amoeba proteus* it has been found[4] that sulfhydryl compounds regulate nuclear growth and fission and in experiments with non nucleated blue green algae, a stimulating influence on cell multiplication has been observed [5]

The demonstration of the physiological significance of sulfhydryl compounds is however, in itself, no proof for their vitagen nature This rests with the demonstration that the organism is unable to synthesize a particular sulfur containing compound which may be essential, such as cysteine or the cysteine containing glutathione or any other normal body constituent of this class There is actually ample experimental evidence that cystine is synthesized in the organism[6,7] from methionine [8] On the other hand the essential nature of methionine has been established in the search for the indispensable amino acids (see page 540) and its efficacy is at least partly due to the available methyl group which acts in transmethylations (see page 543) Whether or not the vitagen action of methionine involves a participation of the sulfur in the molecule is not known Future research must decide whether or not sulfur or sulfhydryl compounds belong to the class of vitagens and if the activity is due to the presence of the sulfur group sulfhydryl group or due to the entire molecule

Among the known vitamins is a sulfur containing compound namely,

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[1] W C Rose *Science* 86 298 (1937) W H Griffith *J Nutrition* 21 ?91 (1941)
[2] See the review by T Bersin *Ergeb Enzymforsch* 4 68 (1935)
[3] P Chaix and C Fromageot *Enzymologia* 6 33 (1939)
[4] C Voegtlin and H W Chalkley *U S Pub Health Service Pub Health Repts* 45 3041 (1930) *J Natl Cancer Inst* 1 63 (1940)
[5] F S Hammett and L Walp *Growth* 3 4?7 (1940)
[6] W C Rose *Physiol Rev* 18 109 (1938)
[7] E F Beach and A White *J Biol Chem* 127 8? (1939)
[8] H Tarver and C. L A Schmidt *Ibid* 130 67 (1939)

vitamin $B_1$ While vitamin $B_1$ is not a sulfhydryl compound but a sulfide, the mechanism of the vitamin action is believed to be due to the ability of the vitamin to undergo reversible oxidation reactions with the intermediary formation of a disulfide (see page 143) It is also known that in this case the vitamin action is due to the specific structure of the entire molecule

# PATENT INDEX

# PATENT INDEX

In the following pages United States British German and French patents dealing ith vitamins are listed They are divided according to subject matter and arranged imerically under each subject The essential data of each patent are given including ie number of the patent and the country in which it issued (U S for United States for Great Britain G for Germany and F for France) This is followed by the date i which the patent was issued The inventor and the assignment recorded at the date issue are indicated A short abstract of the claim of the patent follows

In addition to patents from the United States Great Britain Germany and France a w patents from other countries such as Austria Australia (Austral ) Belgium (Belg ) anada (Can ) Denmark (Den ) Japan (Jap ) Norway (Norw ) Russia (Russ ) weden (Swed ) and Switzerland (Swiss) are mentioned because of their outstanding terest

Abbreviations have been used for a number of industrial organizations

Ciba stands for Chemical Industries of Basel
DuPont stands for E I du Pont de Nemours & Co
Glaxo stands for Glaxo Laboratories Ltd
Hilger stands for Adam Hilger Ltd
I C I stands for Imperial Chemical Industries
I G stands for Interessen Gemeinschaft der Farben industrie
Pfizer stands for Charles Pfizer & Co Inc
Squibb stands for E R Squibb & Son

## VITAMINS GENERAL

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| S 883 174 | Mar 31 1908 | F S Davidson and W P Burra | Extracts from hops and yeasts by steam ng under pressure followed by press ng to separate the con densed liquid extract |
| S 1 461 703 | July 10 1923 | D Ch dlow | Pre rving the vitam n content of cere ls by heating to 132 C |
| S 1 474 746 | Nov 20 1923 | G S Ward | Bread enriched with all vitamins of natural origin |
| S 1 479 418 | Jan 1 1924 | N Min nberg | Separation of vitamins of whole grain cooking of the starchy ex tracted material addition of the vitamin extract to the cooked ex tracted material and drying |
| S 1 479 502 | Jan 1 1924 | G Heffele | Yeast mixture as vitamin food |
| S 1 480 520 | J n 8 1924 | J R Eoff | Vitamin elix r in w ne from ye t or yeast concentrate |
| S 1 481 671 | Jan 22 19 4 | T J Allen | Vitamin-containing food by fermen tation of yeast |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 488 815 | April 1 1924 | I F Harris | Vitamin extract from yeast |
| U S 1 502 563 | July 22 1924 | W P Heath | Preserving vitamins in bread by working in an atmosphere of $CO_2$ |
| U S 1 526 032 | Feb 10 1925 | J A Wessener | Vitamin-containing food by mixing steep water with yeast followed by evaporation |
| U S 1 538 366 | May 19 1925 | R Willstätter and H Sobotka | Yeast rich in vitamins |
| U S 1 540 883 | June 9 1925 | I F Harris | Vitamin preparations especially water soluble vitamins are preserved from deterioration by admixture with non hygroscopic sugars |
| U S 1 541 263 | June 9 1925 | C Hoffman H D Grigsby and N M Cregor | Vitamin containing food ingredients from rice polishings wheat bran or cereal germ by cooking or by proteolytic enzymatic action followed by saccharification by malt diastase |
| U S. 1 552 176<br>B 242 645 | Sept 1 1925<br>Nov 8 1925 | H Liebers | Production of nutrient materials rich in vitamins by mixing yeast with concentrated extracts of germinated cereals. |
| U S 1 552 549<br>B 258 704<br>F 611 760 | Sept 8 1925<br>Oct 6 1926<br>Oct 11 1926 | B L Eicher | Charcoal tablets containing medicinals and fish oils |
| U S 1 568 196 | Jan 5 1926 | O Stiner A Hauswirth and A Gams | Vitamin malt preparation |
| U S 1 574 776 | Mar 2 1926 | R Willstätter and H Sobotka | Yeast preparation rich in vitamins |
| U S 1 589 192 | June 15 1926 | T C Manchester | Preservation of the vitamin content of milk by replacing the air in receptacles containing the milk with an inert gas such as $CO_2$. |
| U S 1 590 837 | June 29 1926 | H Liebers | Addition of yeast to cheese to increase its vitamin content. |
| U S 1 624 154 | April 12 1927 | M Winckel | Vitamin preparation from fermented skim milk and yeast. |
| U S. 1 633 711 | June 28 1927 | R K. Prince<br>Ass to Vitamin Food Co | Process for sealing vitamins A and D from cod liver oil into a mixture of yeast or other vegetable products with a gum solution |
| U S 1 690 091<br>B 289 798<br>G 545 268<br>F 655 799 | Oct. 30 1928<br>April 30 1927<br>May 1 1928<br>April 27 1928 | J K. Marcus | Separation of vitamins A D and E along with unsaponifiable matter from oils by saponification and extraction with ethylene dichloride |
| U S. 1 708 914 | April 9 1929 | B Dass<br>Ass to Ellis Foster Co | Incorporation of dried yeast into peanut butter |
| U S 1 722 175 | July 23 1929 | W S. Bowen | Spray desiccation for material containing vitamins |
| U S. 1 746 657 | Feb 11 1930 | W J Kemp | Preparation of vitamin-containing tomato juice |
| U S. 1 753 531 | April 8 1930 | R. K. Prince<br>Ass to Vitamin Food Co | Endocrine gland substances are mixed with vitamin preparations. |
| U S 1 756 574 | April 29 1930 | J Takamine J Takamine and N Fujita<br>Ass to Takamine Ferment Co | Vitamin product from propagated fungi such as aspergillus |

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|---|---|---|---|
| U S 1 764 085 | June 17 1930 | H Placek<br>Ass to G E Conkey Co | Vitamin containing granular products from cod liver oil yeast or soy bean meal and paraffin |
| U S. 1 775 966<br>U S. Reissue 18 542<br>U S. 1 775 967<br>U S Reissue 18 523<br>B 256 765<br>B 274 051 | Sept 16 1930<br><br>July 26 1932<br>Sept. 16 1930<br><br>July 12 1932<br>Aug 19 19 6<br>July 8 1920 | E H Miles and G Reilly | Preparation of vegetable extracts rich in vitamins by mixing the juices of vegetables containing vitamins and preservatives adding acid material and heating the mixture to accomplish hydrolysis |
| U S 1 796 077 | Mar 10 1931 | H Iscovesco<br>Ass to Health Products | Process of isolating lipoids by acetone extraction |
| U S. 1 845 813<br>U S. 1 918 983 | Feb 16 1932<br>July 18 1933 | R K Prince<br>Ass to Vi Foods Co | Vitamin concentrate from a blend of different vegetables |
| U S 1,861 677<br>B 268 655<br>G 486 228 | June 7 1932<br>Sept 24 1926<br>Sept 25 1926 | L A Agopian | Vitamin concentrate from fruits or vegetables by precipitation with a Pb- or Cu salt followed by extraction of the precipitate |
| U S 1 879 762 | Sept 27 1932 | F W Nitardy<br>Ass to Squibb | Vitamin-containing tablets are provided with a coating containing an antioxidant such as hydroquinone |
| U S. 1 886 931 | Nov 8 1932 | E R Alexander<br>Ass to Vitamin Co of America | Mixing of vitamins with citrus fruit masses |
| U S 1,896 490<br>U S 1 896,5 0<br>U S. 1,896 521 | Feb 7 1933<br>Feb 7 1933<br>Feb 7 1933 | E Komm | Vitamin-containing food product from wheat germ |
| U S. 1 913 515<br>B 317 554<br>F 655 181 | June 1 1933<br>May 25 1928<br>June 2 1928 | C Schmitt | Vitamin extract from cotton seeds |
| U S 1 919 297<br>G 508 503 | July 25 1933<br>Oct 23 1928 | W Kropp F Lange and A Bohne<br>Ass to Winthrop | Stable aqueous solutions of fat soluble vitamins by using water soluble amides of lower fatty acids as solvents for the vitamins |
| U S. 1 929 786 | Oct 10 1933 | A E Meyer<br>Ass to Chappel Brothers | Isolation of chondroitin compounds from cartilage |
| U S. 1 942 943 | Jan. 9 1934 | C F Schnabel | Poultry feed rich in vitamins from grass |
| U S 1 964 867<br>Continuation of U S. 1 633 711 | July 3 1934 | L B Allyn<br>Ass to Vitamin Food Co | Granules of dried yeast impregnated with a vitamin A-containing oil and paraffin |
| U S. 1 965 355 | July 3 1934 | C L Patterson | Vitamin-containing foodstuff from milk and yeast |
| U S. 1 975 169 | Oct 2 1934 | A B O Norrbin | Extraction of water soluble vitamins by dilute acids and addition of a substance soluble in acid solution which precipitates upon neutralization and carries the vitamins in the precipitate |
| U S. 1 984 858<br>B 441 513<br>F 782,314 | Dec 18 1934<br>Jan 21 1936<br>June 3 1935 | C L Barthen<br>Ass to Health Products Co | Countercurrent extraction of vitamins from an aqueous alkaline soap |
| U S. 1 988 677<br>U S. 1 988 678 | Jan 22 1935<br>Jan. 22 1935 | G D Arnold | Portable apparatus for drying vegetable matter while preserving the vitamin content |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 999 789 | April 30 1935 | E Schmierer<br>Ass to Baker Perkins Co | Apparatus for extracting vitamin containing juices from fruits and vegetables in a vacuum |
| U S 2 006 700 | July 2 1935 | G C Supplee G E Flanigan and R C Bender<br>Ass to The Borden Co | Method for separating vitamins from casein |
| U S 2 017 942 | Oct 2 1935 | S Botcharsky<br>Ass to S Teitelbaum | Fat soluble vitamins are obtained from animal or vegetable material by plasmolysis followed by extraction |
| U S 2 041 056 | May 19 1936 | W L Fleisher | Vitamin containing compounds are injected into freshly baked products such as bread |
| U S 2 041 1[illegible] | May 19 1936 | C Hoffman<br>Ass to Ward Baking Co | Cereal food is enriched in vitamins by adding natural vitamin containing ingredients to dough |
| U S 2 052 218 | Aug 25 1936 | C Dickens | Vitamin product by concentrating milk |
| U S 2 052 219 | Aug 25 1936 | C Dickens | Vitamin concentrate from asparagus |
| U S 2 060 389 | Nov 10 1936 | A I Wigelsworth | Apparatus for drying organic materials while preserving their vitamin content |
| U S 2 065 332 | Dec [illegible] 1936 | C W Kirby and C N Frey<br>Ass to Standard Brands | Vitamin product from yeast |
| U S 2 072 402 | Mar 1937 | F Kretschmer<br>Ass to Krause Medico | Vitamin containing food product from unripe apples |
| U S 2 128 845 | Aug 30 1938 | R P Myers and S M Weisberg<br>Ass to Sealtest System Laboratories | Whey is fermented with a lactose fermenting microorganism to produce a product rich in vitamins |
| U S 2 1[illegible]8 946 | Sept 6 1938 | M B Katzman | Tetraphosphates of aliphatic hydroxy compounds are used to retard rancidification of vitamin containing preparations |
| U S 2 13[illegible] 6[illegible]6 | Oct 11 1938 | A R Smith<br>Ass to Combustion Engineering Co | Flash drying of materials containing vitamins |
| U S 2 133 36[illegible] | Oct 18 1938 | C F Schnabel<br>Ass to American Dairies | Vitamin concentrate from grass juices |
| U S [illegible] 141 4[illegible] | Dec 27 1938 | C Waizmann | Vitamin products from yeast and vegetable material |
| U S 2 14[illegible] 344 | Jan 31 1939 | I Draisbach<br>Ass to Hall Laboratories | Vitamin in oil water emulsions using an alkali metaphosphate as the emulsifying agent |
| U S 2 1[illegible]1 644 | Mar 1 1939 | H C Stephens<br>Ass to Natural Food Products | Deaeration of fruit juices to preserve the vitamin content |
| U S 2 157 75[illegible] | May 9 1939 | C G Harrel and A W Lindert<br>Ass to Pillsbury Flour Mills Co | Addition of an oil soluble dye to vitamin preparations to facilitate the degree of mixing with other feed |
| U S [illegible] 1[illegible]7 144 | July [illegible] 1938 | R W Barton and W M Cox<br>Ass to Mead Johnson | Vitamin emulsions containing glycerol a mono oleic acid ester of diglycerol and sucrose |

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|---|---|---|---|
| U S 170 ?0 | Aug 22 1939 | J A Raynolds Ass to Atlantic Coast Fisheries Co | Gelatine capsules for vitamin bearing liquids |
| U S 2 183 053<br>B 489 970<br>B 490 001 | Dec 12 1939<br>Aug 2 1938<br>Aug 2 1938 | H F Taylor Ass to Atlantic Coast Fisheries Co | Incorporation of vitamins in gelatine droplets |
| U S 2 186 82 | Jan 9 1940 | W W Cowgill | Apparatus for drying vitamin containing foods in film form |
| U S ? 188 319 | Jan 30 1940 | G F Siemers Ass to McKesson & Robbins and to Vital | Composition of vitamin A with an adsorbate of vitamins $B_1$ and B |
| U S 2 189 438 | Feb 6 1940 | L H Smith and C F Schnabel Ass to American Dairies | Vitamin-containing fodder by mixing dried greens with a milk product |
| U S ? 194 6 | Mar 26 1940 | C M Porter L V Porter and E *Hurlock* | Food product rich in vitamins from lactobacillus cultures |
| U S 2 195 595 | Apr 1 2 1940 | F W Nitardy Ass to Squibb | Stabilization of fatty materials containing vitamins by the addition of an anti acid substance such as MgO |
| U S 2 19? 90<br>U S 2 06 113 | April ? 1940<br>July 2 1940 | F W Nitardy Ass to Squibb | Vitamin tablets containing Ca gluconate or Ca phosphate |
| U S 2 197 095<br>B 4 8 399<br>B 473 506<br>F 797 654 | April 16 1940<br>Dec 18 1936<br>Oct 14 1937<br>May 1 1936 | B Cuenod Ass to Soc d étude et applications industrielles | Addition of vitamins to milk preparations |
| U S 2 ?00 319 | July 2 1940 | F Getz | Addition of vitamins to ground coffee by a spray process. |
| U S ? ?30 517 | April 1 1941 | F J Cahn and B R Harris Ass to Emulsol Co | Emulsification of vitamins with a reaction product of an anhydride of a carboxylic acid ester of a hydroxy polycarboxylic acid and a partial ether or ester of an aliphatic polyhydroxy-compound |
| B 19? 343 | April 5 1923 | P M Travis and C Claba | Vitamin-containing milk by addition of fish oils malt extracts and yeast |
| B 199 043 | June 11 1923 | P Farup | Addition of alcohol to fish oils Also addition of plant extracts |
| B 217 8<br>G 431 158 | Mar 5 19?3<br>Sept ?0 19?6 | Kellogg Co | Vitamin extracts from bran milk or other sources are added to dough or bread preparations |
| B 20 3 | Sept 17 19?3 | J Schmidt | Vitamin-containing materials such as *yeast* are added to products similar to malt extracts |
| B 2 5 | May ?9 1923 | Fleischmann Corp | Addition of vitamins to yeast nutrient solutions |
| B 2 6 549 | Dec ?1 19 3 | Mellemeuro Pae sk | Vitamin preparations are added to cheese |
| B 237 ?42 | July 16 19 4 | O Mustad and Sön | Margarine and other edible fatty materials are mixed with substances rich in vitamins in the presence of an inert gas |
| B 49 74?<br>G 456 387<br>F 604 077 | April 8 1926<br>Feb 21 1928<br>April 8 19?6 | P M W Greick | Vitamin-containing food from malted grain |

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|---|---|---|---|---|
| B | 254 724 | July 1 1925 | F H Peck | Addition of vitamins to beverages. Also ultraviolet light irradiation of beverages |
| B | 271 626 | July 20 1927 | H Haddan | Vitamin-containing tablets from dried vegetables or fruits |
| B | 280 212 | May 17 1928 | S Grönningsaeter and Fischer Hollinshed Co | Process of transferring vitamins by extracting the vitamins from vitamin-containing material and adding the substance to be vitaminized to the solution containing the extract |
| B | 293 735 | April 11 1927 | Vitamin Food Co | Dried yeast and cod liver oil are mixed with a gum and dried to form an air excluding coating on the granular particles. |
| B | 297,256 | Dec. 14 1927 | J E Nyrop | Production of vitamin-containing powders suitable for adding to margarine by spray-drying emulsions containing cream or skimmed milk |
| B | 315 340 | Jan 10 1928 | W Kollath and H Magistris Ass to L. W Gans A G | Vitamin extract by precipitation and adsorptions |
| B | 320 369 | July 2 1928 | J Korselt | Vitamin solutions from plant juices are treated with an acid Ca salt to remove oxalic acid. |
| B<br>G | 326 742<br>48? 494 | July 25 1929<br>June 18 1921 | Van Den Bergh s Margarine Ges | Incorporation of emulsions of vitamins into margarine. |
| B<br>B<br>F<br>and Add | 328 942<br>362 0?3<br>655 343<br>39 331 | Feb 4 1929<br>Sept 11 1930<br><br>Sept 8 1930 | Matro Ges | Vitamins are extracted from rootlets of grain from malt houses |
| B<br>B<br>F | 340 580<br>360 282<br>681 093 | Nov 24 1928<br>Jan 23 1931<br>Sept 2 1929 | VanHouten & Zoon | Addition of vitamins to chocolate and cocoa |
| B<br>G | 343 086<br>570 158 | Nov 13 1929<br>Nov 14 1929 | A B O Norbin and A Astra Apotekarnas Kemiska Fabrieker | Extraction of water soluble vitamins by diluted acids precipitations and extractions |
| B<br>G<br>F | 346 574<br>519 778<br>696 910 | June 15 1929<br>June 16 1929<br>April 15 1930 | U Brinch H Spehr and C E Sander<br>Ass to Brinch and Spehr | Preservation of vitamins in dried fruits by the addition of acids and salts prior to the drying |
| B | 350 684 | May 14 1930 | E Maybury | Food seasonings and sauces containing vitamins |
| B<br>G<br>G<br>G<br>G<br>G<br>F | 357 732<br>598 027<br>609 744<br>618 899<br>625 075<br>636 434<br>698 375 | Sept 13 1929<br>Jan 3 1935<br>Feb 22 1935<br>Oct 31 1935<br>Feb 3 1936<br>Oct 9 1936<br>July 4 1930 | H Van de Sandt | Addition of vitamins to beer |
| B<br>G<br>G | 362 023<br>501 844<br>647 542 | Dec 9 1931<br>July 9 1930<br>July 7 1937 | Matro Ges | Production of vitamin extracts |
| B | 365 256 | Dec 1 1930 | H Van de Sandt | Dealbuminized vitamin concentrate from yeast tomatoes spinach etc. |

| Patent No | | Date | Patentee | Abstract |
|---|---|---|---|---|
| B | 366 516 | Oct 19 1929 | L Bernardini | Fat and water soluble vitamins from germs and cereals are added to food products |
| B | 367 063 | Nov 29 1929 | H Krönig | Enrichment of vitam ns in beer by the addition of opened yeast |
| B | 367 909 | Dec 3 1929 | Ass to F Lux | |
| B | 368 919 | Jan. 30 1930 | | |
| G | 579 369 | June 24 1933 | | |
| B | 369 633 | Dec 23 1930 | W W Triggs | Vitamin products from citrus fruits |
| F | 709 423 | Aug 6 1931 | Ass to Tropical V tamin Co | liver o ls wheat germ oils etc |
| B | 370 926 | April 6 1932 | J E Nyrop | Vitam n-containing feed for ani mals |
| B | 378 399 | Mar ?4 1931 | L W Mapson J T Mac Curdy H O Nolan and Cam bio Products Ltd | Edible vitamin-containing food products by autolysis or fermenta tion of animal or pl nt material |
| B | 395 957 | July 27 1933 | A R Jahn | Mult ple effect evaporator for the concentration of vitam ns |
| B | 418 214 | Oct 8 1934 | Salsterol Lab | Aqueous vitamin concentrate from raw fresh plant tissue |
| B | 425 998 | Mar 26 1935 | I G | Emulsions of fat soluble vitamins in o l with water and an emulsifying agent such as gelat n gum arabic yolk of egg etc |
| B | 454 528 | Oct 2 1936 | A Nyrop | Vitam n extract from p lped raw material by tr atment with steam under pressure |
| B | 459 467 | Jan 8 1937 | Nyegaard & Co | Vitam ns are ncorporated in $CO_2$ evolving compounds containing sub tances of acid reaction |
| B | 506 09 | May 23 1939 | I G | Manufacture of edible glycerides to which vitam ns may be added |
| G | 362 367 | Oct 7 1922 | T Hamburger | Dry vitamin preparat on from plant juices and calcium lactate |
| G | 39 442 | Mar 21 1924 | Bayer | Food product from plasmol zed yeast and calcium phosphate |
| G | 470 035 | Jan 3 1929 | M Winkel | Vitamin contai ing yoghurt |
| G | 483 394 | Aug 12 1925 | H Aman | E tract on of vitam ns and inositol phosphor c acid |
| G | 489 186 | M y 13 19 6 | O Reinke | Vitamin extract from asparagus |
| G | 49 281 | Feb 20 1930 | J Wolf | Extr ction of vitamins from fi h livers by ac dic solvents |
| G | 499 384 | Aug 1 19?5 | Akt enfabrik z Er eugung von Chemik lien | In the preparation of vitamin ex tracts the starting material is pl smolyzed f llowed by saccharifi cation |
| G | 504 816 | June 7 1922 | Di m lt A G | Vitam n products from yeast |
| G | 505,35? | Aug 18 1930 | P Grube | Vitam n-conta n ng food product from the berr es of S b s au a pa a |
| G | 511 993 | Mar ?8 19?9 | H Jena and J Jen | Add t on of vitamin-contain ng fruit ju ces to cheese |
| G | 516 820 | July 29 1927 | R Neugebau r | Food rich in vitam n from germ nating cereals |
| G | 521 1 6 | Aug 29 1926 | Knoll A G | Purificat on of vit mins by frac tion l d ffusion or dialy is |

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|---|---|---|---|---|
| G | 528 250 | Feb 12 1930 | A Keddi | A vitamin extract from barley to be added to dough |
| G | 532 521 | Mar 20 1927 | H Netzow & Co | Vitamin preparations from irradiated yeast are added to margarine |
| G | 537 057 | May 19 1928 | Enossis S A | An aqueous vitamin extract from cotton seeds is added to milk and milk products |
| G | 549 304 | Feb 26 1930 | H Van de Sandt | Stabilization of beverages to which vitamins have been added by adjusting the pH to about 4 |
| G | 561 686 | April 12 1927 | Vitamin Food Co | Bird food rich in vitamins from yeast cod liver oil and gum |
| G | 565 901 | April 22 1931 | A Hölscher | Vitamin product by fermenting vegetable material containing vitamins with yeast |
| G<br>G<br>G | 57[illegible] 546<br>577 622<br>631 18 | April 13 1933<br>May 11 1933<br>June 15 1936 | H Van de Sandt | Vitamin containing beer |
| G<br>Add to<br>G | 581 143<br><br>561 725 | July 21 1933 | H Kronig | Beer is enriched in vitamin content by adding disintegrated yeast before the end of the fermentation |
| G | 586 589 | Oct 25 1933 | F Weinmann | *d* Glucuronic acid from plant germs by hydrolysis |
| G | 586 959 | Oct 27 1933 | W Lesselberg | Vitamin preparations are mixed with albuminous material and dried |
| G | 618 482 | Sept 9 1935 | H Sander & Co | Food preparations containing extracts of water and fat soluble vitamins |
| G | 623 610 | Dec 30 1935 | K Bodendorf | Food preparation by extracting vegetable matter with cod liver oil |
| G | 623 657 | Jan [illegible] 1936 | W Kropp F Lange and A Bohne<br>Ass to I G | Preparation of water emulsions of fat soluble vitamins by dissolving the vitamins in water soluble ethers or esters of polyhydroxy-compounds followed by incorporation into water |
| G | 626 776 | Mar 2 1936 | Hoffmann LaRoche | Extraction of vitamins with solutions of bile acids followed by separation of the acids |
| G | 642 307 | Mar 1 1937 | F Laquer<br>Ass to I G | Dispersions of fat soluble vitamins in water by means of albuminous materials |
| G | 661 503 | June 20 1938 | P Lindner | Assimilation of alcohol and/or $CO_2$ by yeast or algae to form vitamins |
| F | 694 443 | July 24 19 9 | L M Rayband | Preservation of vitamins in germinated seeds by a coating of sugar chocolate |
| F | 715 445 | Aug 26 1930 | A A Gournier | Food product rich in vitamins from cereals and milk |
| F | 717 067 | May 15 1931 | I G | Dispersion of vitamins in water by the aid of albumins |
| F | 723 537 | July 18 1931 | G Dubois | Extraction and concentration of vitamins |
| F | 735 596 | April 20 193 | Établissements Byla | Food products from yeast |

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|---|---|---|---|
| F 757 454 | Dec 27 1933 | H C Meyers | Food product rich in vitam ns from the germs of grain |
| F 771 059 | Sept 29 1934 | M C Gulbransen | Concentrated vegetable juices are mixed with vitam n containing li quids |
| F 796 101 | Mar 30 1936 | M Ernotte | Vitamin composition containing lecithin sugar and vitamins espe cially vitamin D |
| F 797 180 | April 2? 1936 | E A Barbet | Vitamin enriched drinks by the ad d tion of concentrates from grapes |
| F 797 229 | April 23 1936 | International Vitaerine Iab Co | Fresh foods are dried to preserve their vitamin content by a liquid which causes exosmosis of the juice |
| F 805 598 | Nov 4 1936 | Soc Française Des Sucres | Addition of vitamins to sugar |
| F 810 961 | Apr l 3 1937 | C G V Bouillon | Apparatus for generating vitamins in grain |
| F 833 984 | Nov 8 1938 | Ocean | The vitamins in a purée of paprika are preserved by sealing under vacuum |
| F 845 046 | Aug 9 1939 | G Sandulesco and A Girard Ass to Les Laboratories Fran ça s de Ch miothérapie | Separation of hydroxyl group containing vitamins by transforma t on into water soluble quaternary ammonium compounds |
| Austral 9 933/35 | Jan 16 1936 | Nestlé and Anglo-Swiss Con densed Milk Co | Fat and water soluble vitamins are emulsified in condensed m lk |
| Austri n 121 078 | Aug 15 1930 | E Rein sch | Yeast is enriched in vitamins by ultraviolet rays |
| Austrian 131 289 | July 15 1932 | H Oleoth | Extract on of vitamins from vege table materials or microorganisms in the presence of nascent hydrogen |
| A stri n 137 4 7 | May 11 1934 | M Kle n | Vitamin preparations from cellular vegetable tissue by dialysis followed by precipitation and extract on pro- cedures |
| D n 54 611 | April 19 1938 | H C E Tösch | Vitam n-containing material for coloring margarine is extracted from plants w th o ls |
| Norw 43 80 | Mar 28 1927 | A W Owe | Vitam n extract from vegetable ma terial by saponification and extrac tion |
| Norw 44 019 | Dec 29 1930 | A W Owe | Edible fat is mixed with a vitamin bearing material |
| **Vitamins A and D** | | | |
| U S 1 162 907 | Dec 7 1915 | C Funk | Extraction of vitam ns from cod liver oil w th l gro n or another organic solvent precip tation w th alcohol or acetone |
| U S 1 3 6 968 | Jan 6 19 0 | G D Roger | Oil s separated from fatty mater als e g f om fish l vers by adding sodium chlor de followed by subjec t on to an electr cal d scharge |
| U S 1 368 148 B 137 514 | Feb 8 19 1 Dec 6 1919 | P M Hey rd hl | E traction and purification of o ls e g fish o ls by water |
| U S. 1 519 779 | Dec 16 1924 | E M Johnson | Oil is p essed from f ozen cod livers. |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Vitamins A and D (*Continued*) | | | |
| U S 1 629 074 | May 17 1927 | C Funk and H E Dubin | Vitamins A and D are obtained from fish oils by extraction with acetic acid *saponification and* digitonin precipitation of non vitamin products |
| U S 1 629 618 | May 24 1927 | S Grönningsaeter | Vitamin containing fats are saponified with alcoholic alkali and the vitamins extracted by a vegetable *oil* |
| U S 1 638 700 | Aug 9 1927 | D Molofsky<br>Ass to Silmo Chemical Co | Powdered fish oil product in a non metallic mineral carrier |
| U S 1 649 520 | Nov 15 1927 | C Funk and H E Dubin | Stabilizing vitamin containing oils by mild hydrogenation |
| U S 1 678 454<br>B 208 145<br>B 227 121<br>B 227 122<br>G 484 993<br>F 579 734 | July 24 1928<br>April 3 1925<br>April 3 1925<br>April 3 1925<br>Oct 24 1929<br>Oct 22 1924 | T F Zucker<br>Ass to University Patents Corp | Extraction of liver oils with alcohol saponification of the extract precipitation of the fatty acids as calcium soaps and extraction of the vitamins from the soaps |
| U S 1 715 945<br>G 472 814 | June 4 1929<br>Oct 11 1924 | A W Owe | Vitamin containing oils are saponified with an alkaline earth hydroxide and extracted with an edible fat |
| U S 1 725 964 | Aug 27 1929 | F W Nitardy<br>Ass to Squibb | Vitamin bearing oils from fish livers by heating under subatmospheric *pressure* |
| U S 1 753 790 | April 8 1930 | K Kawai | Partial saponification of cod liver oil |
| U S 1 786 095<br>B 220 697<br>F 566 695 | Dec 23 1930<br>Aug 14 1924<br>Feb 18 1924 | K. Takahashi<br>Asst to Z H R Kenkyujo<br>*Japan* | Extraction of fat soluble vitamins from fish oils by saponification and *precipitation of* the fatty acids with an alkaline earth metal followed by extraction of the vitamins with an organic solvent |
| U S 1 805 593<br>B 266 905<br>G *501 834* | May 19 1931<br>Mar 2 1926<br>*Nov 4 1924* | A W Owe | Vitamin extracts from marine oils by saponification followed by extraction *with a vegetable oil* |
| U S 1 845 370 | Feb 16 1932 | T B Wagner | Emulsions of cod liver oil with a calcium phosphorus compound derived from steepwater of corn |
| U S 1 879 734 | Sept 27 1932 | W G Christiansen and E Moness<br>Ass to Squibb | Extraction of vitamins from a saponified material by means of acetone |
| U S 1 896 185 | Feb 7 1933 | H O Nolan<br>Ass to Ellis Foster Co | Partially hydrogenated cod liver oil containing its original vitamin content |
| U S 1 897 039 | Feb 14 1933 | W G Christiansen W S Jones and E Moness<br>Ass to Squibb | Precipitation of fatty acids in the *saponification mass of cod* liver oil as the Al salt |
| U S 1 919 369 | July 25 1933 | H A Holaday and A Black<br>Ass to Squibb | Saponification of vitamin containing oils and extraction with ether acetone or dichloro ethyl ether |
| U S 1 925 489 | Sept 5 1933 | E Langfeldt and R Hellerud | Apparatus for the extraction of vitamins from solutions containing water soluble soaps by means of a vaporized solvent |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Vitamins A and D (*Continued*) | | | |
| U S 1 935 042 | Nov 14 1933 | A Black<br>Ass to Squibb | Refining the vitamin-containing unsaponifiable part of fish oils by dissolving in alcohol or dioxane and washing with a vegetable oil |
| U S 1 947 315 | Feb 13 1934 | W O Snelling | Vitamin concentrate from fish oils by saponification in pentane with aqueous alkalies. |
| U S 1 947 432 | Feb 13 1934 | R C Huston and H D Lightbody<br>Ass to State Board of Agriculture of Michigan | Preservation of vitamin-containing fish liver oils by the addition of hydroquinone resorcinol or the like |
| U S 1 983 654 | Dec 11 1934 | A Black<br>Ass to Squ bb | Refining the vitamin-containing unsaponifiable matter of o ls by treatment with carbon in the presence of an antioxidant |
| U S 1 988 969 | Jan 22 1935 | A F O Germann<br>Ass to S M A Corp | Vitamin A and D concentrate from palm oil by separation of the fraction permanently liquid at low temperature and irradiation or addition of viosterol |
| U S 2 007 108<br>B 463 655 | July 2 1935<br>April 5 1937 | H Bresnick | Vitamin preparation by mixing an edible dehydrated hard vegetable fat with a fatty material of high vitamin content |
| U S 2 0?6 395 | Dec 31 1935 | H P Loomis<br>Ass to Silmo Chem cal Co | Vitamin concentrate from oils by partial saponification |
| U S 2 051,257 | Aug 18 1936 | H N Holmes<br>Ass to Parke Davis | Materials such as fish liver oil carotene or irrad ated ergosterol are stabil zed by adding phospholipides such as lecithin sphingomyelin etc |
| U S 2 067 279 | Jan 12 1937 | F W Nitardy and W S Jones<br>Ass to Squibb | V tamin-contain ng oil is extracted from fish livers the protein of which has been coagulated by means of ethylene chloride or other chlorinated solvents. |
| U S 2 090 738 | Aug 24 1937 | A O Tischer<br>Ass to Eastman Kodak | Purification of vitamin A and D containing natural oils by extraction of the impurities with an aldehyde having a furane nucleus |
| U S 2 136 453 | Nov 15 1938 | H M Merker<br>Ass to Parke Davis | Purification of vitamin-containing ether e tracts from fish oils by means of sodium aluminum silicate. |
| U S 2 136 481 | Nov 15 1938 | F H Young and H D Robinson<br>Ass to Abbott | Vitamins A and D concentrate by partial saponificat on of l vers |
| U S. 2 150 315<br>B 506 730 | Mar 14 1939<br>June 5 1939 | A. E Briod and B R East<br>Ass to National Oil Products | Process for emulsifying vitamins A and D concentrates with cream or evaporated milk |
| U S. 2 161 882 | June 13 1939 | James A. Patch | Method of extracting vitamins from fish liver oils by saponifying adding organic solvents to produce a homogeneous solution and subsequently add ng excess water |
| U S 2 173 629 | Sept 19 1939 | N A Milas<br>Ass. to Research Corp | Process for the isolation of substantially pure vitamins A and D from fish liver oils by a series of fractional crystallizations |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Vitamins A and D** (*Continued*) | | | |
| U S 2 179 917 | Nov 14 1939 | H Brinton | Addition of iodine to vitamin A and D containing oils as a stimulating effect for poultry |
| U S 2 201 061<br>2 201 062<br>2 201 063<br>2 201 064 | May 14 1940 | B H Thurman<br>Ass to Refining Inc | Stabilization of fats and oils by a vegetable phosphatide |
| U S 2 207 712 | July 16 1940 | J G Blaso<br>Ass to Natural Vitamins Co | Concentration of vitamins in oils by hydrogenation of the oil removal of the hardened material and recovery of the vitamin containing mother liquors |
| U S 2 255 875<br>B 535 014 | Sept 16 1941<br>Mar 26 1941 | L O Buxton and F J Simons<br>Ass to National Oil Products | Purification of fat soluble vitamin containing oils by treatment with activated carbon |
| U S 2 258 671 | Oct 14 1941 | L O Buxton<br>Ass to National Oil Products | Refinement of vitamin containing oils by treatment with sugars |
| U S 2 258 672 | Oct 14 1941 | L O Buxton and H B Colman<br>Ass to National Oil Products | Process for refining vitamin containing oils by treatment with aliphatic aldehydes |
| U S 2 258 673 | Oct 14 1941 | L O Buxton<br>Ass to National Oil Products | Process for refining vitamin containing oils by treatment with edible gum |
| U S 2 266 719 | Dec 16 1941 | L O Buxton and F J Simons<br>Ass to National Oil Products | Process for refining a fat soluble vitamin containing material with an adsorbent in a non polar solvent containing about 10% of a polar solvent |
| U S 2 266 830 | Dec 23 1941 | H F Taylor A W Wells and V A Nedzvedsky<br>Ass to Atlantic Coast Fisheries | Vitamin concentrate from oils by saponification followed by extraction |
| B 3 075 | Feb 5 1915 | Boehringer | Extraction of vitamins from cod liver oil with ligroin and precipitation with phosphotungstic acid and extraction with acetone |
| B 207 545 | Dec 5 1923 | P M Heyerdahl | Liver oils are added to food products *e g* to chocolate or plant juices |
| B 214 238 | April 9 1923 | F W Nitardy<br>Ass to Squibb | Packing of cod liver oil and similar food substances in the presence of an inert gas |
| B 217 363 | April 5 1923 | T W F Clark and F F Pearson & Co | Improvement in odor and flavor of fish oils or their unsaponifiable part by passing a stream of inert gas through the oil followed by heating |
| B 227 474 | Jan 11 1924 | D A Hansen | Oils are obtained from fish livers by passing inert gas through the material at 40–50 C |
| B 266 139 | Mar 2 1926 | A W Owe | Vitamin concentrates are added to oils and the mixture subjected to a mild refining treatment |
| B 267 410 | Mar 17 1927 | H Iscovesco and A B Adams | Extraction of vitamins from cod livers by saponification followed by extraction of the vitamins with a solvent |

| Patent No | | Date | Patentee | Abstract |
|---|---|---|---|---|
| Vitamins A and D (*Continued*) | | | | |
| B | 289 187 | Feb 7 1927 | F H Carr<br>Ass to British Drug Houses | Extraction of livers with an oil |
| B | 293 777 | April 5 1927 | L H Lampitt and J H Bushill<br>Ass to J Lyons & Co | Fats of high vitamin content from frozen viscera by heating with alkali solution and separating the fat |
| B | 305 929 | Feb 11 1928 | K Helholt | Vitamin containing oils are added to non saponifiable oils saponified extracted with a solvent and added to another oil |
| B | 316 656 | Aug 2 1928 | H F Taylor<br>Ass to Atlantic Coast Fisheries | Fish livers are preserved by an oil solution of NaF |
| B<br>B | 334 950<br>361 343 | Nov 5 1930<br>Dec ° 1931 | E Langefeldt and E Hellerud | Extraction of soap solutions from liver oils by means of benzene introduced in vapor form |
| B | 381 342 | Oct 1? 1932 | K Kawai | Oil from fish livers by treatment with dilute alkali and centrifuging the separated oil |
| B | 392 060 | Oct 1 1930 | I G | Vitamin-containing hydrogenated fish oils are made aromatic by adding peppermint oil anise oil or the like |
| B | 433 930 | Aug 2? 1935 | Ferrosan A ( | Isolation of the vitamin-containin unsaponifiable fraction of oils |
| B | 433 938 | Sept 4 1935 | I G | Use of hydroxy alkyl-ethers and alkoxy ethyl ethers of polyhydroxy compounds as water miscible solvents for fat soluble vitamins. |
| B | 441 545 | Jan 16 1936 | B A Rewald | Extraction of vitamins from fish tissue |
| B | 461 °0 | Feb 1? 1937 | Aarus Oliefabriek and C E Christensen | Isolation of vitamins from oils and fats by simultaneous extraction and saponification |
| B<br>G | 494 26<br>664 021 | Oct 24 1938<br>Aug 18 1938 | E Auhagen<br>Ass to I G | Manufacture of stable vitamin A and vitamin D preparations by mixing oily vitamin preparations with fat-containing plant material *e g* germinated wheat |
| B | 500 770 | Feb 15 1939 | G H Lubarsky<br>Ass to Vitamol Inc | Manufacture of vitamin compositions for feeding poultry and live stock by emulsifying an oil concentrate of vitamins A and D in molasses |
| B | 506 730 | June 5 1939 | A E Briod & B R East | Vitamin concentrates and use thereof in making food products particularly milk products |
| B | 533 323 | Feb 11 1941 | G H Lubarsky<br>Ass to Vitamol Inc | Vitamin composition by mixing a fish liver oil vitamin concentrate emulsified in molasses with an animal feed meal followed by drying |
| B | 535 383 | April 8 1941 | L O Buxton<br>Ass to National Oil Products | Vitamin concentrate from the unsaponifiable fraction of marine oils by adsorption followed by elution |
| B | 537 403 | June 20 1941 | L O Buxton and E J Simons<br>Ass to National Oil Products | Refining of fat soluble vitamin containing oils by activated carbon |

| Patent No | | Date | Patentee | Abstract |
|---|---|---|---|---|
| **Vitamins A and D** (*Continued*) | | | | |
| B | 541 003 | *Nov 10 1941* | *H P Kaufmann* | Refinement of vitamin-containing oils by chromatographic *adsorption* |
| G | 452 646 | Nov 15 1927 | I G | Liver oils are refined by treatment with alcohols in the absence of oxygen |
| G | 468 301 | Nov 4 1924 | A W Owe | Vitamin extracts *e g* carotene by saponification neutralization and removal of solvent |
| G | 492 281 | June 3 1927 | J Wolf | Extraction *of livers or fish liver oil* with an acidic agent having a pH between 3 and 6 |
| G<br>G | 448 870<br>496 597 | Sept 1 1927<br>April 26 1930 | H Sander & Co | *Vitamin containing margarine from* cod liver o l |
| G<br>*G* | 560 146<br>*567 648* | Feb 26 1926<br>*Mar 31 1927* | A G f Medizinische Produkte | Extraction of vitamins from oils by means of hot alcohol and/or acetone |
| G | 568 901 | Oct 12 1930 | J D Riedel E de Haen | Petroleum ether extraction of the saponifiable part of cod liver oil |
| G | 593 395 | Feb 26 1934 | Pentosin Werke | Emulsion from liver oil lime and calcium saccharate |
| G | 692 711 | Sept 27 1940 | F Unger<br>Ass to Heyl & Co and to F Unger | Extraction of vitamins A and D by means of esters of low M W acids and low M W alcohols |
| Belg | 430 869 | Nov 30 1938 | M Vermeulen | Natural products containing vitamins are saponified directly and extracted |
| Can | 259 310 | Mar 30 1926 | P M Heyerdahl | Incorporation of vitamins in margarine by mixing cod liver oil with *olive oil prior to the addition* to margarine |
| Can | 374 395 | June 14 1938 | H N Brocklesby<br>*Ass to the Fisheries Research* Board of Canada | Process of preparing natural fish liver *oils* of high vitamin A and D potency by digestion of the liver proteins with pepsin peptization of the digested material with a mild alkali and *separation of the* liberated oil |
| Norw | 34 895 | May 8 1922 | T B Lexow | Margarine is enriched in vitamins *by the addition of animal fats* high in vitamin content such as cod liver oil |
| Norw | 41 688 | July *27 1925* | Smoerfabrikken *Flora* | *Fish liver oil to* be added to margarine is treated to cover the unpleasant taste *e g* by add ng aromatic substances and emulsification |
| **Distillation Procedure** | | | | |
| U S<br>B | 1 260 072<br>120 8?0 | Mar 19 1918<br>Dec 31 1917 | W P Schuck<br>Ass to *Superior Oil & Process* Co | Purification of oils by distilling off *impurities* |
| U S<br>U S reissue | 1 925 559<br>20 ?0 | Sept 5 1933<br>April *26 1938* | K C D Hickman<br>*Ass to Eastman Kodak* | Vacuum distillation of fish o ls |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Distillation Procedure (*Continued*) | | | |
| U S ?113 30<br>U S ?1 4 87J<br>B 479 802<br>F 8 5 979<br>F 834 540 | April 5 1938<br>July 26 1938<br>Feb 11 1938<br>Mar 18 1938<br>Nov 23 1938 | K C D Hickman<br>Ass to Ea tman Kodak | Proces for the separation of ita mins A and D from natural oils by molecular d stillation in the pres ence of dyes as indicator for the distillation points of the vitamins |
| U S ?117 80 | May 17 1938 | K C D Hickman<br>Ass to Eastman Kodak | Apparatu for molecular dist llation of organic substances in degassed condition |
| U S 2 117 803 | May 17 1938 | F C D Hickman<br>Ass to Eastman Kodak | Apparatus for molecular distillation by spraying the compound to be distilled under vacuum into the hot vapor of a distilling substance |
| U S 2 1?6 466 | Aug 9 1938 | K C D Hickman<br>Ass to Eastman Kodak | High vacuum short path distilla tion of oils containing vitam ns |
| U S 1 6 467<br>B 487 697<br>F 817 030 | Aug 9 1938<br>June 24 1938<br>Aug 24 1937 | K C D Hickman and J C Hecker<br>Ass to Fastman Kodak | Short path high vacuum dist llation and process for removing the distil late by a flow of an inert liquid over the condensing surface |
| U S ?1 8 ?3 | Aug 30 1938 | R C J Fraser<br>Ass to I C I | Fraction l short path dist llation |
| U S 2 136 774 | Nov 15 1938 | K C D Hickman<br>Ass to D stillation Products | Replacing the a r by an inert gas or vapor prior to molecular distillation |
| U S 2 143 587<br>B 452 442<br>G 659 ?17<br>F 802 177 | Jan 10 1939<br>Aug 2? 1936<br>April 28 1938<br>Aug 29 1936 | H I Waterman C van Vlo drop and A VanDijk<br>Ass to I C I | Preparation of vitamin A and D concentrates wh ch are free from ta te and odors by molecular dis tillation of fish oils followed by mild hydrogenation |
| U S 2 144 906 | Jan 24 1939 | H I Waterman and C van Vlodrop<br>Ass to I C I | Flavoring matters and vitamins from butter fat by dist llation |
| U S 2 146 894 | Feb 14 1939 | K C D H ckman<br>Ass to Distillation Products | Separation of sterols and vitamin D from oils by high vacuum distilla t on |
| U S ?1 0 683<br>B 480 885<br>F 812 734 | Mar 14 1939<br>Mar 2 1938<br>May 15 1937 | K C D Hickman<br>Ass to Distillation Products | Dist llation of vitamin-containing o ls in the presence of antioxidants |
| U S ?150 684<br>F 82 978 | Mar 14 1939<br>Mar 18 1938 | K C D H ckman<br>As to D st llation Products | Solid materials containing vitamins are subjected to molecular distilla tion |
| U S 2 150 68<br>B 48? 883 | Ma 14 1939<br>Apr l 6 1938 | K C D H ckman<br>Ass to D stillation l roducts | Apparatus for molec l r d stilla tion |
| U S 2 165 378<br>B 476 134<br>B 482 88<br>B 480 433<br>F 811 766<br>F 825 974 | July 11 1939<br>Dec 2 1937<br>Apr 6 1938<br>Aug 15 1938<br>Apr ?2 1937<br>Mar 18 1938 | K C D Hickman<br>A s to Eastman Kodak and to D stillation Products | D t llat on in the presence of a sub- stance of low volatility such as tripelargonine |
| U S 2 169 195<br>B 481 189<br>G 664 745 | Aug 8 1939<br>Mar 7 1938<br>Sept 5 1938 | K C D Hickman and A O Tischer<br>Ass to D stillat on Products | Est rs of vitam n A and unsatu rated fatty acids having at least 8 carbon atoms |
| U S 2 180 0 1<br>U S 2 210 9 6 | Nov 14 1940<br>Aug 13 1940 | K C D Hickman<br>Ass to Di till tion Prod ct | Method of d st lling fi h oils to yield prod cts of h gh vitamin con tent by subjecting the oil to a de- gas ing oper tion prior to distilla tion |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Distillation Procedure** (*Continued*) | | | |
| U S 2 180 356 | Nov 21 1939 | K C D Hickman<br>Ass to Distillation Products | Distillation of vitamins A and D from fish liver oils |
| U S 2 186 669 | Jan 9 1940 | E W Fawcett and G Burrows<br>Ass to I C I | Apparatus for short path high vacuum distillation of vitamin containing fish oils |
| U S 2 199 994<br>U S 2 199 995 | May 7 1940<br>May 7 1940 | K C D Hickman<br>Ass to Distillation Products | Distillation of fish oils |
| U S 2 [illegible] | June 25 1940 | K C D Hickman<br>Ass to Distillation Products | Concentrates of vitamin A and of vitamins A and D are obtained from naturally occurring animal oils and fats by molecular distillation |
| U S 2 210 927<br>G 700 764<br>F 834 936 | Aug 13 1940<br>Nov 28 1940<br>Dec 6 1938 | K C D Hickman<br>Ass to Distillation Products | Vacuum distillation process |
| U S 2 229 173 | Jan 21 1941 | K C D Hickman<br>Ass to Distillation Products | A concentrate of vitamin A and D in ester form is made by molecular distillation of the crude ester saponification *concentration of the* vitamin and re esterification |
| U S 2 249 524 | July 15 1941 | K C D Hickman and J C Hecker<br>Ass to Distillation Products | Process for removing substances of undesirable odor and taste from vitamin containing oils by short path distillation of the undesirable materials leaving the purified vitamin oil as distillation residue |
| U S 2 249 525<br>B 528 994 | July 15 1941<br>Nov 21 1940 | K C D Hickman<br>Ass to Distillation Products | Purification of vitamin A esters by alcohol extraction of impurities |
| B 415 088<br>G 670 016<br>F 767 191 | Aug 17 1934<br>Jan 10 1939<br>July 19 1934 | F H Carr and W Jewell<br>Ass to British Drug Houses | High vacuum short path distillation of vitamins from unsaponified liver oils |
| B 464 395<br>F 811 920 | April 19 1937<br>April 26 1937 | E W Fawcett and D Whittaker<br>Ass to I C I | Concentration of vitamins by partial saponification followed by high vacuum distillation |
| B 479 816 | Feb 11 1938 | K C D Hickman<br>Ass to Eastman Kodak | The undistilled residue of a molecular distillation is recirculated over the vaporizing element of the distillation apparatus |
| B 48[illegible] 881<br>B 493 948 | April 6 1938<br>Oct 18 1938 | Eastman Kodak | High vacuum short path distillation in the presence of *constant* yield oil |
| B 48[illegible] 549 | May 18 1938 | K C D Hickman<br>Ass to Eastman Kodak | Molecular distillation of unsaturated oils to be used for blending vitamin concentrates |
| B 487 367<br>F 834 375 | June [illegible]0 1938<br>Nov 18 1938 | G G R Smith<br>Ass to Eastman Kodak | Saponification of the vitamin fraction obtained by high vacuum distillation and separation of the unsaponifiable part followed by high vacuum short path distillation |
| B 488 678 | *July 15 1938* | *Eastman Kodak* | *High* vacuum distillation of sterols and vitamins |
| B 489 6[illegible]3 | July 29 1938 | K C D Hickman and A O Tischer<br>Ass. to Eastman Kodak | High vacuum distillation of materials containing sterols |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Distillation Procedure (*Continued*) | | | |
| B 500 195 *Add to* 482 883 | July 29 1937 | Eastman Kodak | Apparatus for high vacuum distillation |
| B 501 841 | Mar 2 1939 | J G Baxter Ass to Eastman Kodak | Distillation of hydrocarbons and vitamins from marine animal oils |
| B 508 469 | June 27 1939 | K C D Hickman Ass to Kodak Ltd | Isolation of vitamin A vitamin A esters and vitamin D from fish oils by molecular distillation |
| B 532 770 | Jan 30 1941 | K C D Hickman Ass to Distillation Products | Short path high vacuum distillation of vitamins |
| B 539 089 | Aug 27 1941 | K C D Hickman Ass to Distillation Products | Extraction of fish tissues with a solvent of a vapor pressure lower than vitamins followed by high vacuum distillation |
| F 825 406 | Mar 3 1938 | Eastman Kodak | Process for treating vitamin A consisting in the esterification of the distillate obtained by the molecular distillation of an animal oil containing vitamin A |
| F 8?5 973 | Mar 18 1938 | Eastman Kodak | *Cholane derivatives* particularly sterols are concentrated by high vacuum short path distillation |
| F 834 93? F 834 937 | Dec 6 1938 Dec 6 1938 | Eastman Kodak | Method and apparatus for short path high vacuum distillation of vitamins A and D |

VITAMINS A

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Provitamins A | | | |
| U S 1,328 278 | Jan 20 1920 | N A Gavin | Extraction of palm oil by cooking centrifuging screening and pressing |
| U S 1 953 607 | April 3 1934 | H N Holmes and H M Leicester Ass to S M A Corp | Carotene from green plant material by alkali hydrolysis followed by chloroform extraction |
| U S 1 967 121 | July 17 1934 | H N Holmes and H M Leicester Ass to S M A Corp | Carotene from carrots by cooking and acetone extraction |
| U S 1 978 981 | Oct 30 1934 | H M Barnett Ass to S M A Corp | Carotene from palm oil by precipitation with iodine |
| U S 1 988 031 | Jan 15 1935 | H M Barnett Ass to S M A Corp | Carotene from carrot oil or carrot powder by extraction |
| U S 2 029 722 | Feb 4 1936 | V Jersey Ass to S M A Corp | Refining carotene containing oils by separating the free fatty acids present as alkali salts |
| U S 2 031 991 | Feb 25 1936 | O Ungnade and W F Richards Ass to S M A Corp | Acetone extraction of carotene from dry soaps |
| U S 2 032 006 | Feb 25 1936 | R J Cross Ass to S M A Corp | Addition of water binding materials to carotene-containing soaps and extraction of carotene from the dry material |
| U S 2 03? 16? | Feb 5 1936 | H M Barnett W O Frohring and A F Germann Ass to S M A Corp | Carotene from carrots or spinach by benzene extraction crystallization of the main amounts of carotene and incorporation of the mother liquor into oil |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Provitamins A (*Continued*) | | | |
| U S 2 131 394 | Sept 27 1938 | W H Test | Concentrate of pigments from vegetable origin by extraction with an organic solvent from an acidified aqueous solution or suspension |
| U S 2 170 872 | Aug 29 1939 | D D Peebles | Macerated fresh plant materials are digested with alkali under pressure followed by solvent extraction |
| B 364 113 | Sept 24 1930 | P S Voigt | Provitamin A and Vitamin C are extracted from lemon peel with alcohol |
| G 67 683 | Jan 7 1933 | R Kuhn | Separation of alpha and beta carotene by two methods 1 chromatographic adsorption and 2 precipitation with iodine |
| G 685 390 | Dec 16 1939 | T Buhr and W Schoenenberger<br>Ass to W Schoenenberger | Provitamin A containing plant juices by centrifuging the plant juice heating the liquid to coagulate and filtering and adding the sediment from the centrifugation to the liquid |
| Hung 116 057 | Mar 16 1937 | Ocean Magyar Konzervyar | Carotene is mixed with pulp of paprika and added to food products |
| Isolation | | | |
| U S 2 076 901<br>G 634 760 | April 13 1937<br>Sept 2 1936 | F Laquer<br>Ass to Winthrop | Preparation of vitamin A by direct saponification of fish livers with aqueous alcoholic caustic alkali metal hydroxide followed by extraction |
| U S 2 111 049 | Mar 15 1938 | H N Holmes<br>Ass to Parke Davis | Purification of vitamin A by chromatographic adsorption |
| U S 2 125 215<br>B 500 087 | July 26 1938<br>April 25 1938 | A D Barbour<br>Ass to Ontario Research Foundation | Process for the production of a vitamin containing fish oil partially hydrogenated in the presence of a highly active nickel catalyst |
| B 43 907 | Dec 10 1925 | Aarhus Oliefabrik and K H Hansen | Incorporation of vitamin A into oil by mixing a vitamin A containing soap from saponified oil with oil and separating the aqueous soap layer |
| B<br>F | 83 265<br>622 912 | Feb 2 1928<br>June 9 1927 | K Kawai | 50-75% Saponification of cod liver oils |
| B 306 881<br>F 670 114 | May 19 1930<br>Feb 21 1929 | T Shimizu | Precipitation of vitamin A from aqueous solutions by means of bile acids |
| B 393 883<br>C 612 369 | June 15 1933<br>April 23 1935 | Hoffmann La Roche | Purification of crude vitamin A preparation by freezing out impurities and precipitating the vitamin A with water The precipitates are filtered through a layer of solid carbon dioxide |
| B 401 095 | Nov 9 1933 | Abbott | Vitamin A and D concentrates are obtained from fish liver oils by steaming the livers and extracting the oil with an organic solvent |
| B 434 432<br>C 636 227<br>I 768 217 | Sept 2 1935<br>Sept 17 1936<br>Aug 2 1934 | K Ritsert<br>Ass to Merck | Purification of vitamin A in tuna fish liver oil by saponification at room temperature followed by ether extraction |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Isolation (*Continued*) | | | |
| B 465 547 | May 10 1937 | K Kawai | Vitamin concentrates from fish oils by saponific tion followed by ex traction with a fatty o l |
| B 500 645 | Not accepted | Aktiebolaget Separator Ass to Bergedorfer Eisen werk A G Astra Werke | Process and apparatus for the ex traction of oil from fish liver by breaking up the cell structure with acids or alkali without dissolving the oil and separating the oil by centrifugal treatment |
| G 468 301 | Nov 10 1928 | A W Owe | Partial saponification of plant m terial while extracting pro/itamin A |
| G 540 701 | Dec 24 1931 | J A de Loureiro | Obtaining vitamin A and vitam n D separately from cod l ver o l by ex traction with acetic ac d followed by petroleum ether extraction of the acetic ac d extr ct Vitamin A is claimed to be in the petroleum ether while vitamin D is in the remaining acetic acid |
| G 660 621 | June 3 1933 | Hoffman La Roche | Separation of components of vita min A by chromatographic adsorp tion |
| G 681 730 | Sept 29 1939 | R Rosenbusch and G Rev erey Ass to Riedel de Haën | Isolation of vitamin A from fish oils by s ponificat on in the presence of minute amounts of water extrac tion of the solid saponification mass with acetone removal of the ace- tone by d stillation and ether ex traction of the residue |
| G 697 762 | Oct 22 1940 | E J Wolf Ass to Nordmark Werke | Separation of vitamin A from ani mal tissues by enzymes followed by extraction with organic solvents |
| F 847 816 | Oct 17 1939 | M Vermeulen | Concentrat on of vitam n A (or pro- vitamins A) by direct sapon fica tion in the absence of oxygen ex traction of the saponification mass with ether freezing out sterols and distilling off the ether The resi dual product is claimed to contain from 3,200 000 to 3 600 000 I U vitamin A/gr |
| Jap 128 808 | Feb 15 1939 | K Kituta | Concentration of vitamin A of fish liver o l by incomplete saponifica tion (50–55%) and s multaneous ex traction |
| Synthesis | | | |
| U S 1 999 110<br>B 418 723<br>G 601 070 | April 23 1935<br>Oct 30 1934<br>Aug 16 1934 | L Ruzicka Ass to Ciba | Synthesis of tetrahydro-v tamin A |
| U S 2 175 843 | Oct. 10 1939 | R Kuhn and C Grundmann Ass to W nthrop | Polyene carboxylic acids and esters and manufacture thereof |
| U S 2 233 375<br>G 696 084 | Feb 25 1941<br>Aug 15 1940 | R Kuhn and C J O R Morris Ass to Winthrop | Condensing beta ionylidene acetal dehyde in the presence of a second ary amine w th beta methyl-croton aldehyde to form ε-(beta ionyli dene) beta methyl sorbin aldehyde and reducing the aldehyde group of the latter compound by an alumi nate of a secondary alcohol |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Synthesis** (*Continued*) | | | |
| U S 2 239 491 | April 22 1941 | R Kuhn and C J O R Morris<br>Ass to Winthrop | 5 (2 Methyl 6 6 dimethyl cyclohexenyl 1') 3 methyl 2 4-*pentadienal is claimed* as an intermediate in the synthesis of vitamin A and its preparation from 4 (2 methyl 6 6 dimethyl cyclohexenyl 1) 2 methyl 1,3 butadiene 1-carboxylic acid esters is *also claimed* |
| B 510 540 | Aug 2 1939 | I M Heilbron and J W Batty | Condensation of citral with beta methyl croton aldehyde |
| G 683 030 | Oct 27 1939 | R Kuhn and K Wallenfels<br>Ass to I G | Production of polyene compounds *e g* 1 30 diphenylpentadecaene by converting polyene alcohols *into* thio or seleno-aldehydes and removing from 2 molecules of the latter sulfur or selenium |
| **Analysis** | | | |
| U S 2 065 953 | Dec 29 1937 | F Twyman and D H Follet<br>*Ass to Hilger* | Photometric apparatus for estimating substances such as cod liver oil vitamin A concentrates and fruit juices which have characteristic selective radiation absorption |
| U S 2 123 573 | July 12 1938 | R L McFarlan and J W Reddie<br>Ass to United Drug Co | Apparatus for measuring the concentration of vitamin A by light of 330 mμ |
| F 760 676 | Feb 28 1934 | Hilger | Determination of vitamin A by light of 328 mμ |
| **Derivatives and Utilization** | | | |
| U S 2 183 084 | Dec 12 1939 | S Reynolds<br>Ass to Atlantic Coast Fisheries | Composition of gelatine with vitamin A etc |
| U S 2 198 214 | April 23 1940 | S Musher<br>Ass to Musher Foundation | Stabilizing alfalfa against oxidation for the retention of its carotene content during drying and storage by the use of a sugar and a phosphatide or $H_3PO_4$ in small proportions |
| U S 2 218 591 | Oct 22 1940 | H F Taylor<br>Ass to The Atlantic Coast Fisheries | Dispersion of vitamin A in gelatine solution addition of glycerine and subsequent drying |
| U S 2 218 592 | Oct 22, 1940 | H F Taylor<br>Ass to Atlantic Coast Fisheries | Substantially dry vitamin A preparations are made *by dispersing* the oil containing the vitamin in a matrix composed of a gelable colloid *e g* gelatine and an invert sugar *e g* molasses honey etc |
| B 491 212 | Aug 24 1938 | S Reynolds<br>Ass to Glaxo | Process for the production of droplets of gelatine containing vitamin A |
| B 503 517 | Not accepted | J Verne and C Mille | Process of protecting fatty substances from becoming rancid by the addition of carotenoids |
| B 507 471 | June 12 1939 | Eastman Kodak | *Retarding oxidation of* animal and vegetable oils by the addition of a small quantity of a fraction possessing antioxidant properties taken from the first 20% distillate obtained by a high vacuum short path distillation of vegetable oils. |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Derivatives and Utilization (*Continued*) | | | |
| G 658 957 | April 20 1938 | I G | Preparation of an emulsion of fat soluble \ itam n concentrates from fish livers |

## VITAMIN B COMPLEX

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 058 927<br>G 266 211<br>B 5 322 | April 15 1913<br>Feb 6 1911<br>Nov 12 191 | J Tsuzuki Japan | Isolat on of vitamin B concentrates from rice bran by alcohol extrac tion |
| U S 1 162 908 | Dec 7 191ɔ | C Funk | Extract on of vit mi from yeast rice bran etc by addit on of phos photungstic acid and extraction with acetone |
| U S 1 173 317 | Feb 29 1916 | A Seidell | Vitamins from yeast by autolysis and adsorption on fuller s earth fol lowed by elut on with d lute acid and alcohol |
| U S 1 235 198 | July 31 1917 | A Gams and B Schreiber | Vitamin concentrate from yeast rice bran beans etc by precipita tion of inactive materials with lead salts and precip tation of the active material with an alkaloid precip tant such as phosphotungstic ac d |
| U S 1 431 5?5<br>B 186 633 | Oct 10 192<br>Oct ° 1922 | C Hoffm n H D Grigsby and N M Cregor<br>Ass to Ward Baking Co | Addit on of vitamin B containing substances such as defatted rice polishings to dough |
| U S 1 474 029 | Nov 13 19 3 | I F Harris | Alcohol extraction of vitamins from yeast boiled in dilute acetic acid |
| U S 1 617 70 | Feb 15 1927 | P Caccia | Extraction of vitamin B with alco-hol precipitation w th ethylene di chloride and crystalliz t on in the pr ence of sulfuric cid |
| U S 1 737 79 | Nov 26 1930 | L Wallerstein<br>Ass to Wallerstein & Co | Manufacture of extracts rich in vitamin B from malt and wheat germs |
| U S 1 842 933 | Jan 26 1932 | B W Dedr ck | Water extraction of the residues ob tained by mak ng flour from wheat followed by f ltration and recovery of the water soluble materials |
| U S 1 893 977<br>B 373 0 8 | Jan 31 1933<br>Sept 1 1931 | J W Dressel | Liver concentrate by e tract on with water |
| U S 2 006 0 3 | June 25 1935 | F Lange and L Taube<br>Ass to Winthrop | Preparations containing vit min B from yeast extract |
| U S 2 006 699 | July 2 193ɔ | G C Supplee and G E Flanigan<br>Ass to The Borden Co | Recovery of vitamins $B_1$ and $B_2$ from milk |
| U S 2 095 638 | Oct 12 1937 | G A Jeffreys | Liquid b nder for food products rich in vitamin B f om fermented material |
| U S 2 184 748<br>B 428 044 | Dec 26 1939<br>Apr 130 193 | R P Light and C N Frey<br>Ass to Standard Brands | Preparat on of v tam ns $B_1$ and $B_2$ from yeast by plasmolysis coagula tion of the protein materi l and con centration of the water solution |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 193 876 | Mar 19 1940 | B Maizel<br>Ass to Vico Products Co | Isolation of the vitamin B cor from yeast by means of 90% m or ethyl alcohol distilling off of the solvent to separate gu constituents or precipitating by alkali earth materials and ing the vitamin-containing mat with extracted yeast or with co lated egg white |
| U S 2,202 161 | May 28 1940 | C S Miner<br>Ass to Commercial Solvents | Recovery of vitamin B compon from fermentation of molasses butyl alcohol producing bacteri |
| U S 2 202 307 | May 28 1940 | L E Boober | Extraction of the vitamin B c plex |
| B 103 294<br>F 485 649 | Jan 4 1917<br>Jan 24 1918 | Ciba | Extraction of vitamins from husks yeast etc by alcohol precipitation of inactive produ by lead salts |
| B 345 669<br>G 566 174 | Nov 22 1929<br>Oct 10 1929 | I G | Aqueous solution of yeast rich vitamin B for beverages |
| B 376 149<br>Jap 91 250 | Nov 10 1931<br>April 28 1931 | S *Ota* and K *Umeda*<br>Ass to K Beer | Pressed yeast is fermented wi cane sugar etc The product is e tracted with alcohol to yield a n trient material containing vitam B |
| B 434 058 | Aug 26 1935 | W W Triggs | Concentrate of vitamins B fro whey |
| B 477 528 | Dec 30 1937 | Standard Brands | Manufacture of a product contain ing vitamins of the B-complex b extraction of seeds or grain germs |
| B 485 079<br>F 824 837 | May 10 1938<br>Feb 17 1938 | I G | Plant growth stimulant containin vitamins of the B-complex |
| B 486 054 | May 30 1938 | Eli Lilly | Solution of a vitamin of the B complex in a polyhydric alcohol |
| B 536 510 | May 16 1941 | Standard Brands | Yeast is grown on a culture medium containing the vitamins $B_1$ $B_6$, inositol pantothenic acid and biotic acid (extracted from sheep liver) |
| B 538 191 | July 24 1941 | Emulsions Process Corp | Extraction of the vitamins of the B-complex from yeast after break ing down the yeast cells by applica tion of pressure followed by a sud den release of the pressure |
| B 539 825 | Sept 25 1941 | Standard Brands | Improvement in the propagation of yeast by the addition to the culture medium of vitamins $B_6$ and $B_1$ (or a pyrimidine and thiazol compound) and additional growth promoting factors |
| G 295 361 | Mar 25 1914 | Boehringer | Purification of phosphotungstate precipitates of vitamins B by ace tone extraction |
| G 607 512<br>G 634 969 | Dec 29 1934<br>Sept 10 1936 | P György R Kuhn and T Wagner Jauregg<br>Ass to I G | Manufacture of vitamins of the B complex by adsorption followed by elution with amines or ammonia |

| PATENT NO | DATE | PATENTEE | ABSTRACT |
|---|---|---|---|
| G 630 772 | June 5 1936 | I G | Isolation of vitamin $B_4$ by adsorption elution and precipitation methods making use of the strong bluish fluorescence char cten tics of vitamin B |
| G 646 548 Continuation of G 630 772 | June 17 1937 | I G | Elution of vitamin B by alkali and by amines Vitamin $B_4$ according to this patent has the formula $C_{11}H_{14}N_4OS$ and seems to resemble vitamin $B_1$ and thiochrome rather than the description of vitamin $B_4$ |
| G 672 078 Continuation of 661 929 and 670 74? | Feb 18 1939 | I G | Method of concentrating growth promoting compounds of the b os type |
| G 703 400 | Mar 7 1941 | Ass to G Henning | Adenosinphosphoric acid from adenosin and phosphoric acid by yeast |
| Jap 91 202 | April 25 1931 | S Izume M Sato I Seto Ass to the South Manchurian Railway Co | The alcoholic extract from soy bean is freed from impurities and contains large amounts of vitamin B |
| Jap 101 137 | May 16 1933 | K Taguchi Ass. to K Katakura | Rice bran or the l ke is extracted with methanol to yield a substance rich in vitamin B |

VITAMIN $B_1$—THIAMIN

| | | | |
|---|---|---|---|
| Isolation | | | |
| U S 1 869 721<br>U S 1 889 427<br>B 354 421<br>F 714 416 | Aug 2 1932<br>Nov 29 1932<br>Aug 13 1931 | B Sure | Purification of crude vitamin $B_1$ concentrates by dissolving in acetic acid precipitating impurities with acetone and adsorbing the vitamin on charcoal |
| U S 1 937 671 | Dec 5 1933 | A Seidell | Elimination of non vitam nic substances from vitamin $B_1$ concentrates by benzoylation |
| U S 1 990 961 | Feb 12 1935 | E H Stuart Ass to Eli Lilly | Removal of vitamin $B_1$ from an adsorbate by solutions of mineral acids |
| U S 2 00? 519 | May 28 1935 | R J Block and G R Cowgill Ass to S J Dannenberg | Purification of vitam n $B_1$ concentrates by oxidation of impurities |
| U S 2 015 876 | Oct 1 1935 | B Sure and E H Stuart Ass to B Sure | Concentration of vitamin B from acid alcoholic extracts from rice brans by adsorption on active carbon |
| U S 2 049 988 | Aug 4 1936 | R R Williams and R E Waterman Ass to Research Corp | Elut on of the fuller s earth adsorbate of vitamin $B_1$ with an acid polynitrogenous alkaloid salt e g with quinine sulfate solution |
| U S 2 114 775<br>B 497 081<br>F 818 702 | April 19 1938<br>Not accepted<br>Oct 2 1937 | L R Cerecedo | Isolation of v tamin $B_1$ from natural sources by adsorption on zeolite followed by elution Further purification by precipitation of the vitamin as silver compound or silicotungstate |
| B 390 378 | April 6 1933 | S J Dannenberg | Vitamin B concentrate by extracting the vitamin at pH 10–13 followed by an acid extraction |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| *Isolation (Continued)* | | | |
| G 311 074 | Jan 12 1916 | Ciba | Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts |
| G 3?0 765 | April 28 1920 | R Bosshard and F Hefti | Vitamin $B_1$ concentrates from plant material e g rice brans yeast etc by total hydrolysis with dil mineral acids at 80 C |
| G 359 878 Continuation of G 311 074 | Sept 28 1922 | Ciba | Hydrolysis of the starting material with enzymes |
| Jap 109 268 | Jan 22 1935 | R Otake | Isolation of crystallized vitamin $B_1$ from yeast by adsorption on acid clay extraction with $Ba(OH)_2$ and precipitation with a silver salt. |
| **Synthesis** | | | |
| U S 2 127 446<br>B 500 519<br>G 676 980<br>F 831 110 | Aug 16 1938<br>Feb 10 1939<br>June 16 1939<br>Aug 23 1938 | M Klingenfuss<br>Ass to Hoffmann LaRoche | Synthesis of vitamin $B_1$ by condensation of 2 methyl-4 amino-5-thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy-3-chlorotetra hydro-furane |
| U S 2 166 233 | July 18 1939 | E R Buchman<br>Ass to Research Corp | Process of preparing 2 methyl-6-amino-5-pyrimidyl bromacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin $B_1$ therefrom by condensation with 4 methyl 5 beta hydroxy ethyl thiazole |
| U S 2 184 720 | Dec 26 1939 | T Matukawa and M Ohta<br>Ass to Kabusiki Kaisha Takeda Chobei Shoten | Synthesis of vitamin $B_1$ from 2 methyl 4 amino 5 formamino-methyl pyrimidine and gamma gamma diaceto gamma mercapto-propyl alcohol |
| U S 2 209 244<br>B 456 735<br>B 471 416<br>G 685 032<br>F 816 432 | July 23 1940<br>Nov 11 1936<br>Aug 30 1937<br>Dec 11 1939<br>Aug 7 1937 | H Andersag and K Westphal<br>Ass to I G | (1) Synthesis of vitamin $B_1$ by condensation of 2 methyl-4 amino-5-thio formylamidomethyl pyrimidine with 2 keto-3 halogeno-pentanol 5 or its acetate<br>(2) Synthesis of vitamin $B_1$ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl 4 amino 5 halogeno methyl pyrimidine<br>(3) Synthesis of vitamin $B_1$ by condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyano-butyric acid with acetamidine |
| U S 2 235 862<br>B 507 918<br>G 669 187<br>Continuation of G 681 638 | Mar 25 1941<br>June 22 1939<br>Dec 19 1938 | O Zima<br>Ass to Merck | Synthesis of vitamin $B_1$ by condensation of 2 methyl-4 amino-5-oxy methyl pyrimidine HCl with 4 methyl 5 beta hydroxy ethyl thiazole HCl |
| U S 2 252 921 | Aug 19 1941 | Z Földi and A Cerecs | Synthesis of vitamin $B_1$ from 2 methyl 4 amino 5 thioformamidomethyl pyrimidine and 2 methyl 2 hydroxy 3 halogeno-tetrahydrofurane in the presence of salts of weak organic bases and strong acids |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| ynthesis (Contin ed) | | | |
| B 496 776<br>F 834 68 | Dec 2 1938<br>Nov 29 1938 | Research Corp | Synthesis of vitamin B by conden sation of 2 methyl 4 amino 5-bromo-methyl pyrimidine HBr with 4 methyl 5 (beta hydroxy ethyl) thiazole |
| B 53 013 | Jan 15 1941 | Standard Brands and Inter national Yeast | The vitamin $B_1$ content of yeast is increased by growing the yeast in a wort to which has been added a pyrimidine compound with or with out a thiazole compound 2 Methyl 5 ethoxymethyl 6-amino-pyrimidine and 4 methyl 5-beta hydroxy-ethyl thiazole are specifically mentioned as additive agents |
| G 681 638<br>Cont nuation of<br>G 669 187 | Sept 27 1939 | O Zima<br>Ass to Merck | Synthesis of vitamin $B_1$ by conden sation of 2 methyl-4 amino-5-al koxy methyl pyrimidine hydrochlo-ride with the hydrochloride of 4 methyl 5 beta hydroxy ethyl thiazole. |
| G 703 775<br>Addition to<br>G 685 03 | Mar 15 1941 | H Andersag and K. Westphal<br>Ass to I G | Synthesis of vitamin $B_1$ accord ing to G P 685 032 but using 5-amino-alkyl thiazole compounds for the condensation followed by con version into vitamin $B_1$ |
| G 705 432<br>Addition to<br>G 685 032 | April 28 1941 | K Westphal and H Andersag<br>Ass to I G | Synthes s of vitamin $B_1$ by conden sation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl-4 amino-5-methyl-pyrimidine the 5-methyl group of which is substi tuted by a reactive group (Example benzol sulfonic acid ester of the 5-hydroxy methyl derivative) |
| Synthesis of the Thiazole Part | | | |
| U S 2 123 653<br>B 496,801<br>G 684,587<br>F 831 111 | J ly 12 1938<br>Dec 6 1938<br>Dec 1 1939<br>Aug 23 1938 | Hoffmann LaRoche | 2 Methyl 2 alkoxy 3 chloro-tetra hydro-furane from alpha-ace-tyl alpha chloro butyrolactone and an aliphatic primary alcohol |
| U S 2 133 969<br>B 472 459<br>G 673 174<br>G 675 617<br>F 803 495 | Oct 7 1938<br>Sept 17 1937<br>Mar 17 1939<br>July 21 1939<br>Oct. 1 1936 | E R B chman<br>Ass to Research Corp | 4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto gamma halogeno-propanol an thio-formamide |
| U S 2 134 015 | Oct. 25 1938 | R R W'lliams<br>Ass to Research Corp | Compos tion of matter claims for methyl 5 beta hydroxy ethy thiazole and its salts |
| U S 2 139,570<br>B 466 751<br>G 704,296<br>F 811,224 | Dec 6 1938<br>Nov 13 1936<br>Mar 26 1941<br>April 9 1937 | H Andersag and K. Westphal<br>Ass to Winthrop | Condensation of gamma-brom gamma aceto propanol with a rhodanide followed by treatment to form methyl thiazolyl 5 esters |
| U S 2 160,867<br>G 670 131 | June 6 1939<br>Jan. 12 1939 | O Hromatka<br>Ass to Merck | 4 Methyl beta ethyl thiazole from geno gamma-aceto-propyl formamide and sulfide |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Isolation** (*Continued*) | | | |
| G 311 074 | Jan 12 1916 | Ciba | Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts |
| G 320 785 | April 28 1920 | R Bosshard and F Hefti | Vitamin $B_1$ concentrates from plant material e g rice brans yeast etc by total hydrolysis with dil mineral acids at 80 C |
| G 359 878<br>Continuation of<br>G 311 074 | Sept 28 192[illegible] | Ciba | *Hydrolysis of the starting* material with enzymes |
| Jap 109 268 | Jan 2[illegible] 1935 | R Otake | Isolation of crystallized vitamin $B_1$ from yeast by adsorption on acid clay extraction with $Ba(OH)_2$ and precipitation with a silver salt |
| **Synthesis** | | | |
| U S 2 127 446<br>B 500 519<br>G 676 980<br>F 831 110 | Aug 16 1938<br>Feb 10 1939<br>June 16 1939<br>Aug 23 1938 | M Klingenfuss<br>Ass to Hoffmann LaRoche | Synthesis of vitamin $B_1$ by condensation of 2 methyl 4 amino 5 thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy 3-chloro tetra hydro-furane |
| *U S 2 168 233* | *July 18 1939* | *E R Buchman*<br>Ass to Research Corp | *Process of* preparing *2 methyl-6* amino-5 pyrimidyl bromacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin $B_1$ *therefrom by condensation with* 4-methyl 5 beta hydroxy ethyl thiazole |
| U S 2 184 720 | Dec 26 1939 | T Matukawa and M Ohta<br>Ass to Kabusiki Kaisha Takeda Chobei Shoten | Synthesis of vitamin $B_1$ from 2 methyl 4 amino 5 formaminomethyl pyrimidine and gamma gamma-diaceto gamma mercaptopropyl alcohol |
| U S 2 [illegible]09 244<br>B 456 735<br>B 471 416<br>G 685 032<br>F 816 43[illegible] | July [illegible]3 1940<br>Nov 11 1936<br>Aug 30 1937<br>Dec 11 1939<br>Aug 7 1937 | H Andersag and K Westphal<br>Ass to I G | (1) Synthesis of vitamin $B_1$ by condensation of *2* methyl 4 amino-5 thio formylamidomethyl pyrimidine with 2 keto-3 halogeno pentanol 5 or its acetate<br>(2) Synthesis of vitamin $B_1$ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl 4 amino 5 halogeno methyl *pyrimidine*<br>(3) Synthesis of vitamin $B_1$ by condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyanobutyric acid with acetamidine |
| U S 2 235 862<br>*B 507 918*<br>G 669 187<br>Continuation of<br>G 681 638 | Mar 25 1941<br>*June 2[illegible] 1939*<br>Dec 19 1938 | O Zima<br>*Ass to Merck* | Synthesis of vitamin $B_1$ by conden*sation of 2 methyl* 4 amino 5 oxy methyl pyrimidine HCl with 4 methyl 5 beta hydroxy ethyl thiazole *HCl* |
| U S 2 25[illegible] 921 | Aug 19 1941 | Z Földi and A Gerecs | Synthesis of vitamin $B_1$ from 2 methyl *4* amino *5 thioform*amidomethyl pyrimidine and *2* methyl 2 hydroxy 3 halogeno tetrahydrofurane in the presence of salts of weak organic bases and *strong* acids |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Synthesis (*Continued*) | | | |
| B 496 7?6<br>F 834 68 | D c 2 1938<br>Nov ?9 1938 | Rese rch Corp | Synthesis of v tamin B by conden sation of 2 methyl 4 am no 5-bromo methyl pyrim dine HBr with 4 methyl 5 (beta hydroxy ethyl) thi zole |
| B 53 013 | Jan 15 1941 | Standard Brands and Inter n tional Yeast | The vitamin $B_1$ content of ye st is increased by growing the yeast in a wort to which has been added a pyrim dine compound with or with out a thiazole compound 2 Methyl 5 ethoxymethyl 6 amino-pyrimidine and 4 methyl 5-beta hydroxy ethyl th azole are specifically mentioned as additive agents |
| G 681 638<br>Continuation of<br>G 669 187 | Sept ?7 1939 | O Z ma<br>Ass to Merck | Synthesis of vitamin $B_1$ by conden sat on of 2 methyl 4 amino-5 al koxy methyl pyrim d ne hydrochlo-ride w th the hydrochloride of 4 methyl 5 beta hydroxy ethyl thiazole |
| G 703 775<br>Add tion to<br>G 685 032 | Mar 1? 1941 | H Andersag and K Westphal<br>Ass to I G | Synthes s of vitam n $B_1$ accord ing to G P 685 032 but using 5-amino alkyl th azole compounds for the cond nsat on followed by con version into vit min B |
| G 705 432<br>Addit on to<br>G 685 032 | April 28 1941 | K Westphal and H Andersag<br>Ass to I G | Synthes s of vitamin $B_1$ by conden sation of 4 m thyl 5 beta hydroxy ethyl thiazole with 2 methyl-4 amino-5 methyl pyrimidine the 5 methyl group of which is substi tuted by a reactive group (E ample benzol sulfonic acid ester of the 5-hydroxy methyl derivative) |
| Synthesis of the Thiazole Part | | | |
| U S 2 123 653<br>B 496 801<br>G 684 587<br>F 831 111 | July 12 1938<br>Dec 6 1938<br>Dec 1 1939<br>Aug 23 1938 | Hoffmann LaRoche | - Methyl 2 alkoxy 3 chloro-tetra hydro furane from alpha ace tyl alpha chloro butyrolactone and an al phatic primary alcohol |
| U S 2 133 969<br>B 472 459<br>C 673 174<br>G 67? 617<br>F 803 495 | Oct 25 1938<br>Sept 17 1937<br>Mar 17 1939<br>July 21 1939<br>Oct 1 1936 | E R Buchman<br>Ass to Research Corp | 4 Methyl ? beta hydroxy ethyl thiazole from gamma aceto-gamma halogeno propanol and thio-form mide |
| U S 2 134 015 | Oct ?? 1938 | R R W ll ams<br>Ass to Research Corp | Composition of m tter claims for 4 methyl 5 beta hydroxy ethyl thiazole and its salts |
| U S 2 139 570<br>B 456 751<br>G 704 236<br>F 811 224 | Dec 6 1938<br>Nov 13 1936<br>Mar ?6 1941<br>April 9 1937 | H Andersag and K Westphal<br>Ass to W nthrop | Condensation of gamma bromo gamma aceto propanol esters with a rhodan de followed by acid treatment to form 2 hydroxy-4 methyl th azolyl 5 ethanol esters |
| U S 2 160 867<br>G 670 131 | June 6 1939<br>Jan 12 1939 | O Hromatka<br>Ass to M ck | 4 Methyl ? beta hydroxy ethyl th azole from g mma halo-geno gamma aceto-propyl alcohol formamide and phosphorus penta sulfide |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Isolation** (*Continued*) | | | |
| G 311 074 | Jan 12 1916 | Ciba | Extraction of animal or plant material with dilute alcohol and precipitation of impurities with lead salts |
| G 320 785 | April 28 1920 | R Bosshard and F Hefti | Vitamin $B_1$ concentrates from plant *material e g* rice brans yeast etc by total hydrolysis with dil mineral acids at 80° C |
| G 359 878<br>Continuation of<br>G 311 074 | Sept 28 1922 | Ciba | Hydrolysis of the starting material with *enzymes* |
| Jap 109 268 | Jan 22 1935 | R Otake | Isolation of crystallized vitamin $B_1$ from yeast by adsorption on acid clay extraction with $Ba(OH)_2$ *and* precipitation with a silver salt |
| **Synthesis** | | | |
| U S 2 127 446<br>B 500 519<br>G 676 980<br>F 831 110 | Aug 16 1938<br>Feb 10 1939<br>June 16 1939<br>Aug 23 1938 | M Klingenfuss<br>Ass to Hoffmann LaRoche | Synthesis of vitamin $B_1$ by condensation of 2 methyl 4 amino 5 thioformylamino methyl pyrimidine with 2 methyl 2 alkoxy 3 chloro tetra hydro furane |
| U S 2 166 233 | July 18 1939 | E R Buchman<br>Ass to Research Corp | Process of preparing 2 methyl-6-amino 5 pyrimidyl bromacetic acid by bromination of the corresponding pyrimidine acetic acid and method of synthesizing vitamin $B_1$ therefrom by condensation with 4 methyl 5 beta hydroxy ethyl thiazole |
| U S 2 184 720 | Dec 26 1939 | T Matukawa and M Ohta<br>Ass to Kabusiki Kaisha Takeda Chobei Shoten | Synthesis of vitamin $B_1$ from 2 methyl 4 amino 5 formamino methyl pyrimidine and gamma gamma diaceto gamma mercapto propyl alcohol |
| U S 2 209 244<br>B 456 735<br>B 471 416<br>G 685 032<br>F 816 432 | July 23 1940<br>Nov 11 1936<br>Aug 30 1937<br>Dec 11 1939<br>Aug 7 1937 | H Andersag and K Westphal<br>Ass to I G | (1) Synthesis of vitamin $B_1$ by condensation of 2 methyl 4 amino-5 thio formylamidomethyl pyrimidine with 2 keto 3 halogeno pentanol 5 or its acetate<br>(2) Synthesis of vitamin $B_1$ by condensation of 4 methyl 5 beta hydroxy ethyl thiazole with methyl 4 amino 5 halogeno methyl pyrimidine<br>*(3) Synthesis of vitamin $B_1$ by* condensation of an ester of gamma (4 methyl 5 acyloxy ethyl N thiazolium bromide) alpha cyano-butyric acid with acetamidine |
| U S 2 235 862<br>B 507 918<br>G 669 187<br>Continuation of<br>*G 681 638* | Mar 25 1941<br>June 22 1939<br>Dec 19 1938 | O Zima<br>Ass to Merck | Synthesis of vitamin $B_1$ by condensation of 2 methyl 4 amino-5 oxy methyl pyrimidine *HCl with* 4 methyl 5 beta hydroxy ethyl thia*zole HCl* |
| U S 2 252 921 | Aug 19 1941 | Z Földi and A Gerecs | Synthesis of vitamin $B_1$ from 2 methyl 4 amino 5 thioformamidomethyl pyrimidine and 2 methyl 2 hydroxy 3 halogeno tetrahydrofurane in the presence of salts of weak organic bases and strong acids |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Synthesis (*Continued*) | | | |
| B 496 7?6<br>F 834 68 | Dec 1938<br>Nov 29 1938 | Research Corp | Synthesis of vitamin $B_1$ by conden sation of ? methyl 4 am no 5-bromo-methyl pyrimidine HBr with 4 methyl 5 (beta hydroxy ethyl) th azole |
| B 53? 013 | Jan 15 1941 | Standard Brands and Inter national Yeast | The vitamin $B_1$ content of yeast is ncreased by growing the yeast in a wort to which has b en added a pyrimidine compound with or with out a thiazole compound 2 Methyl 5 ethoxymethyl 6 amino-pyrimidine and 4 methyl 5 beta hydroxy ethyl th azole are spec fically ment oned as add tive agents |
| G 681 638<br>Continuation of<br>G 669 187 | Sept 27 1939 | O Zima<br>Ass to Merck | Synthesis of vitam n B by cond n sation of 2 methyl-4 amino 5 al koxy methyl pyrimidine hydrochlo-r de with the hydrochloride of 4 methyl 5 beta hydroxy ethyl th azole |
| G 703 775<br>Add tion to<br>G 685 032 | Mar 15 1941 | H Andersag and K Westphal<br>Ass to I G | Synthesis of vitamin $B_1$ accord ing to G P 685 032 but us ng 5-am no alkyl th azole compounds for the condensation followed by con version into vitamin B |
| G 705 432<br>Addition to<br>G 685 03? | April 28 1941 | K Westphal an l H Andersag<br>As to I G | Synthesis of vitamin $B_1$ by conden sation of 4 methyl 5 beta hydroxy ethyl thiazole with 2 methyl-4 amino 5 methyl pyrimidine the 5-methyl group of which is substi tuted by a reactive group (Example benzol sulfonic acid ester of the 5-hydroxy methyl der vative) |
| Synthesis of the Thiazole Part | | | |
| U S 2 123 653<br>B 496 801<br>G 684 587<br>F 831 111 | July 12 1938<br>Dec 6 1938<br>Dec 1 1939<br>Aug 23 1938 | Hoffmann LaRoche | 2 Methyl 2 alkoxy 3 chloro tetra hydro furane from alpha ace-tyl alpha chloro butyr l ctone and an al phatic prim ry alcohol |
| U S ? 133 969<br>B 4 459<br>G 673 174<br>G 675 617<br>F 803 495 | Oct .5 1938<br>Sept 17 1937<br>Mar 17 1939<br>July 21 1939<br>Oct 1 1936 | E R Buchman<br>Ass to Re rch Corp | 4 Methyl o b ta hydro y ethyl thiazole f om gamma aceto-gamma halogeno-propanol and thio-form m de |
| U S 2 134 015 | Oct 25 1938 | R R Will ms<br>Ass to Research Corp | Compo ition of matter claims for 4 methyl 5 beta hydroxy ethyl thiazole and its salts |
| U S 2 139 570<br>B 456 751<br>G 704 ?36<br>F 811 224 | Dec 6 1938<br>Nov 13 1936<br>Mar 26 1941<br>April 9 1937 | H Andersag and K. Westphal<br>Ass to Winthrop | Condensation of gamma bromo gamma aceto propanol esters with a rhodanide followed by acid treatment to form 2 hydroxy-4 methyl thiazolyl 5 ethanol esters |
| U S 160 867<br>G 670 131 | June 6 1939<br>Jan 12 1939 | O Hromatka<br>Ass to Merck | 4 Methyl o beta hydroxy thyl thiazole from gamma halo-geno gamma aceto-propyl alcohol formamide and phosphorus penta sulfide |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| *Synthesis of the Thiazole Part (Continued)* | | | |
| U S 2 179 984<br>B 492 637<br>G 678 153 | Nov 14 1939<br>Sept 23 1938<br>July 13 1939 | H Spiegelberg<br>Ass to *Hoffmann LaRoche* | 4 Methyl 5 beta hydroxy ethyl thiazole from the corresponding 2 mercapto derivative by hydrogen peroxide oxidation |
| U S 2 193 858 | Mar 19 1940 | E R Buchman<br>Ass to Research Corp | Composition of matter claims for alpha halogeno alpha aceto γ butyro lactone and method of preparation by halogenation of alpha aceto γ butyro lactone |
| U S 2 194 179<br>Continuation of<br>U S 2 133 969 | Mar 19 1940 | E R Buchman<br>Ass to Research Corp | 4 Methyl 5 beta acetoxy ethyl thiazole from gamma halogeno gamma aceto propylacetate and thio-formamide |
| U S 2 209 092 | July 23 1940 | D Price and I D Pickel<br>Ass to National Oil Products | Preparation of 4 methyl 5 beta amino-ethyl thiazole by brominating levulinic ester reacting the halogenated compound with thioformamide treating the reaction product with ammonia dehydrating the amide thus obtained to the corresponding cyano-derivative and catalytically hydrogenating the cyano-group to an amino-group |
| U S 2 216 574<br>B 472 396 | Oct 1 1940<br>Sept 17 1937 | T D Perrine<br>Ass to Research Corp | Halogeno aceto-propyl alcohol from aceto-propyl alcohol by direct halogenation |
| U S 2 218 349 | Oct 15 1940 | E R Buchman<br>Ass to Research Corp | Composition of matter claims for γ halogeno-γ aceto-propyl alcohol and methods of preparation by halogenation of γ aceto-propyl alcohol |
| U S 2 218 350 | Oct 15 1940 | E R Buchman<br>Ass to Research Corp | Method of producing γ halogeno-γ aceto propyl alcohol by simultaneous hydrolysis and decarboxylation of an alpha halogeno-alpha aceto-γ butyro-lactone |
| U S 2 223 885 | Dec 3 1940 | I R Buchman<br>Ass to Research Corp | gamma Halogeno gamma aceto propyl esters and their preparation by halogenation of a gamma aceto propyl ester |
| U S 2 263 014 | Nov 18 1941 | W Scott<br>Ass to Wingfoot Corp | Preparation of thiazoles from 2 mercapto-thiazoles by pyrolysis |
| U S 2 267 313 | Dec 23 1941 | J R Stevens and G A Stein<br>Ass to Research Corp | Production of gamma aceto propyl ether |
| B 490 571<br>F 826 067 | Aug 17 1938<br>Mar 22 1938 | Research Corp | Process for the manufacture of γ halogeno γ aceto propyl alcohol and of alpha halogeno-alpha aceto-γ butyro lactone from alpha aceto-γ butyro-lactone |
| G 664 789 | Sept 5 1938 | A Wenz<br>Ass to Merck | 4 Methyl 5 beta hydroxy ethyl thiazole by halogenation of alpha aceto-butyrolactone followed by condensation with thio-form amide |

| Patent No. | Date | Patentee | Abstract |
|---|---|---|---|
| **Synthesis of the Thiazole Part** (*Continued*) | | | |
| G 702 831 | Feb 17 1941 | H Andersag and K Westphal Ass to I G | 4 Methyl 5 beta hydroxy ethyl thiazole from gamma aceto-gamma halogeno propyl com pounds and thioformamide fol lowed by saponification |
| G 705 434 | *April 28 1941* | *K. Westphal and H Andersag* Ass to I G | 2 Methyl 4 alkoxy 3 halo-geno-tetra hydro-furane from 1 aceto 1 halogeno 1 alkoxy ethyl acetone and a mineral acid |
| **Synthesis of the Pyrimidine Part** | | | |
| U S 2 153 016 | April 4 1939 | R R Williams Ass to Research Corp | 2 Methyl 5 amino methyl 6 amino-pyrimidine and derivatives |
| U S 2 184 964 | Dec 26 1939 | G A Stein Ass to Research Corp | 2 Methyl 5 chloromethyl 6-amino-pyrimidine from the corre sponding 5 alkoxy compound |
| U S 2 194 190 | Mar 19 1940 | R R Williams Ass to Research Corp | 2 Methyl 5 methyl 4 halo-geno-pyrimidines |
| U S 2,220 243<br>B 478 993<br>G 675 881<br>F 822 533 | Nov 5 1940<br>Jan 28 1938<br>May 20 1939<br>Dec 31 1937 | M Hoffer Ass to Hoffmann LaRoche | 2 Methyl 4 amino 5 thio-formyl amino methyl pyrimidine and its manufacture from 2 methyl 4 amino 5 amino methyl pyrimidine di hydrochloride and potassium di thioformate |
| U S 2 235 638<br>G 667 990 | Mar 18 1941<br>Nov 24 1938 | O Hromatka Ass to Merck | 2 Methyl 4 amino 5 cyano-pyrimidine from amino-methylene malonitrile and acetimino-ethyl ether |
| B 473 193<br>G 670 095<br>F 819 596 | Oct 4 1937<br>Jan 11 1939<br>Oct 21 1937 | H Andersag and K Westphal Ass to I G | 2 Methyl 4 amino 5 amino-methyl pyrimidine from acetamidine and formyl malonic ester |
| B 475 507 | Nov 19 1937 | I G | 4 Amino 5 hydroxy alkyl pyrimidines from the corresponding 5 amino-compound by nitrous acid |
| B 475 5 9<br>G 671 787 | Nov 19 1937<br>Feb 15 1939 | H Andersag and K Westphal Ass to I G | Synthesis of 2 methyl-4 amino-methyl pyrimidine by condensation of acetamidine with alpha-cyano-succinic ester to the corresponding 6 hydroxy pyrimidine derivative and elimination of the 6-hydroxyl group via the chloride |
| B 486 414 | June 2 1938 | Hoffmann LaRoche | Manufacture of 2 methyl-4 amino-5-cyano-pyrimidine from acetimino-ethyl-ether and amino methylene-malonitrile |
| B 496 738 | Dec 2 1938 | Research Corp | 2 Methyl 4 amino 5 hydroxy methyl pyrimidine by condensation of acetamidine with formyl beta ethoxy propionic ester followed by chlorination and replacement of the chlorine by an amino group |
| B 52 531 | June 20 1940 | Merck | 2 Methyl 5 chloro methyl 4 amino-pyrimidine from the corre sponding 5-alkoxy-compound by hydrogen chloride |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Synthesis of the Pyrimidine Part** (*Continued*) | | | |
| B 538 743 | Aug 14 1941 | Chinoin Gyogyszer es Vegyeszeti Termekek | 2 Alkyl 4 amino 5 carbalkoxy pyrimidines from alpha-cyano-beta amidino acrylic acid esters |
| G 670 635 | Jan 23 1939 | O *Hromatka* Ass to Merck | 2 Methyl 4 amino 5 cyano-pyrimidine from alkoxy methylene malonic acid derivatives ammonia and acetimido ethyl ether |
| **Derivatives and Utilization** | | | |
| U S 2 188 323<br>G 704 172 | Jan 30 1940<br>Mar 25 1941 | H Tauber<br>Ass to Merck | Synthesis of cocarboxylase from vitamin $B_1$ pyrophosphate and phosphoric acid |
| U S 2 205 807 | June 25 1940 | J Bjorksten | Composition of matter claims for vitamin $B_1$ and biotin in a solution of a pH below 4 5 for the thiazole and pyrimidine parts of the vitamin $B_1$ molecule either separately or together in the presence of biotin at a pH between 2 and 4 5 as plant growth stimulants |
| U S 2 224 174 | Dec 10 1940 | J Weijlard Ass to Merck | Purification of synthetic cocarboxylase from vitamin $B_1$ by fractional precipitation of the silver salts at various pH |
| G 663 588 | Aug 9 1938 | K Lohmann and P Schuster Ass to I G | Isolation of cocarboxylase |
| Swed 94 746 | Feb 22 1939 | H v Euler and R Vestin | Vitamin $B_1$ phosphate by enzymatic phosphorylation of the vitamin |
| Swiss 197 717 | Aug 1 1938 | Hoffmann LaRoche | HCl salts of vitamin $B_1$ by precipitating a picrate and converting the *latter to the HCl salt* |

VITAMIN $B_2$—RIBOFLAVIN

| | | | |
|---|---|---|---|
| **Isolation** | | | |
| U S 2 139 857 | Dec 13 1938 | H F Seibert Ass to S M A Corp | Precipitation of riboflavin in mixture with a precipitation of lead sulfide followed by extraction of the filtered sulfide |
| U S 2 175 014 | Oct 3 1939 | L E Booher and L T Work | Isolation of riboflavin from adsorbates by elution with water alcohol or acetic acid preferably at an elevated temperature |
| U S 2 186 314 | Jan 9 1940 | S Ansbacher G E Flanigan and G C Supplee Ass to The Borden Co | *Extraction of riboflavin adsorbates* with aqueous acetone |
| U S 2 188 008 | Jan [illegible]3 1940 | S H Lassen Ass to P R Park Inc | Isolation of the vitamins of the B complex and especially of vitamin $B_2$ from fish press water |
| U S 2 222 306 | Nov 19 1940 | A G Atwood | Simultaneous production of alcohol for bourbon and rye whiskies and a riboflavin containing material from grains |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Isolation (*Continued*)** | | | |
| U S 2 239 285 | Apr 128 1941 | G E Flanigan and G C Supplee | Isolation of vitamin B (riboflavin) from concentrates by pre cip tat on of mpurities in an ace tone water solution with ethyl alco hol followed by ether extraction precipitation of foreign matter w th acetone and final cry stall zat on from the concentrated acetone solu tion |
| B 5?4 445 | Aug 7 1940 | N V Organon | Separat on of r boflav n from its phosphoric ac d ester by elution of an adsorbate of these substances with a d lute aqueous solution of an amine or acid amide or by elect ve ad orption of r boflavin from a d lute solut on of an amine or ac d amide |
| B 5?4 515 | Aug 8 1940 | N V Organon | Proce s for tl separation of ribo flavin from its phosphoric acid ester by distr bution between water a water immiscible solvent s ch as a ph nyl sub tituted aliphatic alcohol or a mi ture of a phenol with an ali phatic alcohol or a m xture of a phenol with a hydrocarbon |
| ( 607 806 | Nov ?1 1938 | K Feudenberg Ass to I G | El t on of physiolog cally active ad sorb tes from met l sulfides by ox dation of the sulfides *e g* with $H\ O_2$ |
| Jap 110 8?6 | M y 17 1935 | W N kah ra and B Inukai Ass to R Kenky jo Co | Concentrate of vitam n B by ad sorption followed by enzymat c li gestion of the adsorbate |
| **Synthesis** | | | |
| U S 2 155 555 | Apr l 1939 | P Karrer Ass to Hoffmann LaRoche | Product cl ims for 7 methyl (or 7 alkyl ) 9 (*d l* r b tyl) so-allo azine |
| U S 2 238 874<br>B 441 692<br>F 79 070 | April 15 1941<br>Jan 20 1936<br>Dec 1 193 | R Kuhn<br>A s to I G | Synthe of v tam n $B_2$ and of other iso allo azine der vati e by con densat on of allox n with N mono sub t tuted aromatic o-d am ne especi lly in ac d solution and in the pr sence of bor c acid |
| U S ?,?61 608 | Nov 4 1941 | M Tishler and J W Wellm n Ass to Merck | Synthe of r bofla n by red ctive condens t on of tetr acetyl ribo o-n trile w th 4 , dimethyl n line coupl ng with para nitro phenyl dia onium chlor de and reduction to N tetra acetyl ribityl amino amino-4 5 d methylbenzene fol lowed by condensat on with 5 5 dichlorobarbituric ac d and fin l hydrolysis |
| B 457 984 | D c 10 1936 | Hoffmann LaRoche | Process for the manufact re of iso allo ine derivatives wh ch con si ts in condensing derivatives of o ph nylened m n which possess a hydroxylat d aliphatic s de cha n att ched to an amino group w th alloxan |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Synthesis** (*Continued*) | | | |
| Swiss 187 938 | Mar 1 1937 | Hoffmann LaRoche | 6 Methyl 9 (d ribityl) iso-alloxazine by condensation of 4 methyl 2 amino phenyl ribamine with alloxan |
| **Intermediates for the Synthesis** | | | |
| U S 2 152 662 | April 4 1939 | F P Phelps<br>Ass to the Government of the United States | Manufacture of ribose by hydrolysis of nucleic acids |
| U S 2 159 804 | May 23 1939 | W E Lawson and C P Spaeth<br>Ass to DuPont | N Ribityl 6 nitro 3 4 xylidine from ribitylamine and 6 nitro-3 4 dimethyl-chlorobenzene |
| U S 2 193 433 | Mar 12 1940 | P L Salzberg<br>Ass to DuPont | Composition of matter claims for N ribityl 3 4 xylidine and process of preparation from 3 4 xylidine and ribose followed by hydrogenation |
| U S 2 237 074<br>B 457 178<br>G 677 515 | April 1 1941<br>Nov 23 1936<br>June 27 1939 | P Karrer<br>Ass to Hoffmann LaRoche | 1 Ribitylamino 2 amino 4 5 dimethyl benzene from N ribityl 3 4 xylidine by coupling with a diazonium salt followed by reduction of the azo dye |
| U S 2 250 999 | July 29 1941 | R Pasternack and F V Brown<br>Ass to Pfizer | Composition of matter claims for tetra acetyl (3 4 dimethyl phenyl) ribityl amine and tetra acetyl (3 4 dimethyl 6 carbethoxy amino phenyl) d ribityl amine and process of making these compounds by condensation of tetra acetyl-d ribose with an aromatic amine followed by hydrogenation |
| B 461 245<br>G 642 148 | Feb 8 1937<br>March 4 1937 | R Kuhn and R Ströbele<br>Ass to I G | Condensation of sugars with ortho-nitro-anilines followed by reduction |
| G 664 048<br>Continuation of 642 148 | Aug 19 1938 | R Kuhn and R Ströbele<br>Ass to I G | Reduction of acyl-derivatives of condensation products of ortho-nitro-anilines and sugars followed by saponification |
| G 664 439<br>Continuation of 642 148 | Sept 1 1938 | R Kuhn and R Ströbele<br>Ass to I G | Condensation of ortho-nitro-anilines with sugars in the presence of ammonium halides or amine hydrogen halides |
| G 679 001<br>Continuation of 642 148 | July 27 1939 | R Kuhn and R Ströbele<br>Ass to I G | Reduction of the condensation products of ortho-nitro anilines and sugars in the presence of borates in slightly alkaline solution to the corresponding phenylene diamines |
| **Derivatives and Utilization** | | | |
| U S 2 068 623 | Jan 19 1937 | O Warburg<br>Ass to Schering | Isolation of the yellow oxidation enzyme from yeast |
| U S 2 111 491<br>B 451 938<br>G 666 791<br>G 647 721<br>F 809 884 | Mar 15 1938<br>Aug 10 1936<br>Oct 28 1938<br>Dec 7 1937<br>Mar 11 1937 | R Kuhn F Weygand and H Rudy<br>Ass to Winthrop | Preparation of 5 phosphoric acid ester of vitamin B |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Derivatives and Utilization (*Continued*) | | | |
| U S 2 256 604<br>G 698 815<br>Continuation of<br>G 686 793 | Sept 23 1941<br>Nov 18 1940 | E Auhagen<br>Ass to Winthrop | Water solutions of vitamin $B_2$ using nicotinic acid in the form of its salts or *N* non substituted amide to increase the solubility |
| B *430 571*<br>G 638 822<br>Continuation of<br>*G 638 138* | *June 17 1935*<br>Nov 23 1936 | *O Warburg*<br>Ass to Schering | Cleavage of *the yellow oxidation* ferment with sodium hydroxide at 50–60 C to produce lumi lacto *flavin* |
| B 438 196<br>*Continuation of*<br>411 179 | Nov 7 1935<br>June 7 1934 | G B Walden<br>*Ass to Eli Lilly* | Vitamin B is added to preparations *for the treatment of pernicious* anem a |
| B 451 779<br>G 637 503<br>F 785 490 | Aug 11 1936<br>Oct 29 1936<br>Aug 10 1935 | Sche ng | Prepar tion of the yellow oxidation f rment by precipitation in aqueou solution at pH 4 in the presence of an acetate buffer |
| B 495 718 | Nov 18 1938 | Ass to I G | Pur ficat on of the yellow ox dat on ferment by adsorption followed by elutr ation with solutions of weakly alkal ne reacting phosphates |
| B *504 721*<br>G 672 018 | April 26 1939<br>June 22 1939 | J Eisenbrand and H Picher<br>Ass to I G | Decompos t on of vitam n $B_2$ during sterilization is restrained by adding urea and an acid *e g* HCl to ob*tain a pH below 5 which pH after* sterilization is between 5 and 7 |
| G 632 131 | July 3 1936 | R Kuhn and H Rud<br>Ass to I G | Manufacture of a diacetone vitamin B |
| G 632 366 | July 7 1936 | Schering | Riboflavin phosphoric acid este from the yellow oxidation ferment from aqueous yeast extract |
| G 633,392 | July 25 1936 | H T Theorell<br>Ass to Schering | Riboflavin phosphoric ac d ester from the yellow oxidation ferment by tr tment with methanol |
| G 637 386<br>Continuation of<br>633 392 | Oct 27 1936 | Schering | Purification of the riboflavin phos phoric acid ester by convers on into n alkali earth salt |
| G 638 138<br>Continuation of<br>G 638 822 | Nov 10 1936 | Schering | Cleavage of the yellow oxidation *f rment with sodium hydroxide* |
| G *686 793* | Jan 16 1940 | E Auhagen<br>Ass to I G | Solution of vitamin $B_2$ in mono-V alkyl derivatives of amides of lower fatty ac ds |
| G 687 197 | Jan 24 1940 | Ciba | Preparat on of vitamin $B_2$—phos phoric ac d ester by phosphoryla tion of the vitamin by means of *phosphatases from the mucous* membranes of the intestines (in the presence of phosphates) and in the presence or absence of compounds of the adrenal cortex |
| G 688 047<br>Continuation of<br>G 686 793 | Feb 10 1940 | F Auh gen<br>Ass to I G | Solutions of vitamin $B_2$ n water using phenol or polyphenol sulfon c acid salts to increase the solubility |

## VITAMIN $B_6$—PYRIDOXIN

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 248 078<br>B 534 916 | July 8 1941<br>Mar 21 1941 | S A Harris<br>Ass to Merck | Synthesis of vitamin $B_6$ by reacting ethoxyacetyl acetone and cyanoacetamide to form 3 cyano-4 ethoxy methyl 6 methyl pyridone 2 hydrolyzing to form the lactone of 3 carboxy 4 hydroxymethyl 6 methyl pyridone 2 treating with nitric acid chlorinating and reducing to form the lactone of 3 carboxy 4 hydroxy methyl 5 amino 6 methyl pyridine diazotizing to form the lactone of 3 carboxy 4 hydroxymethyl 5 hydroxy 6 methylpyridine and reducing the latter compound to form vitamin $B_6$ |
| U S 2 250 396<br>G 690 5_5 | July 22 1941<br>Dec 2 1940 | W Salzer<br>Ass to Winthrop | Synthesis of vitamin $B_6$ by degradation of 3 alkoxy *quinaldine* 4 carboxylic acid |
| U S 2 261 188 | Nov 4 1941 | J V Scudi<br>Ass to Merck | Boric acid salts of vitamin $B_6$ |
| U S 2 266 754 | Dec 23 1941 | S A Harris<br>Ass to Merck | Catalytic reduction of 3-cyano 4 ethoxy methyl 5 nitro 6 methyl pyridone 2 and acetylation of 3 cyano 4 ethoxy methyl 5 amino-6 methyl pyridone |
| U S 2 296 16 | Sept 15 1942 | Ass to I G | Fat soluble derivatives of vitamin $B_6$ by acylation |
| B 534 917 | Mar 21 1941 | Ass to Merck | The vitamin $B_6$ base from its hydrochloride |
| B 536 249 | May 8 1941 | Merck | 2 Methyl 3 methoxy pyridine 4 5 dicarboxylic acid ester from the corresponding acid by esterification |
| B 538 000 | July 16 1941 | Merck | Lactones of 2 methyl 3 alkoxy 4 hydroxymethyl 5 carboxy pyridine by alkylating the lactone of 2 methyl 3 hydroxy 4 hydroxy methyl 5 carboxy pyridine |
| G 684 975 | Dec 8 1939 | R Kuhn and G Wendt<br>Ass to I G | Isolation of a vitamin $B_6$ protein symplex from animal or plant material |
| G 702 829 | Feb 17 1941 | K Westphal and H Andersag<br>Ass to I G | Vitamin $B_6$ by degradation of 3 methyl 4 alkoxy isoquinoline |
| G 702 830 | Feb 17 1941 | K Westphal<br>Ass to I G | 2 Methyl 3 methoxy 4 5 bis amino methyl pyrimidine from the corresponding 4 5 dinitrile by catalytic reduction |
| G 704 761 | April 7 1941 | K Westphal<br>Ass to I G | Synthesis of vitamin $B_6$ by the action of nitrous acid on 4 5 bis amino methyl methoxy pyridine |

## VITAMIN $B_8$

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 970 175<br>B 396 135 | Oct 9 1934<br>Aug 3 1933 | C L Lautenschläger and F Lindner<br>Ass to Winthrop | Production of muscle adenosine phosphoric acid from yeast adenosine triphosphoric acid by hydrolysis |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 098 976 | Nov 16 1937 | S L Ruskin<br>Ass to F R Ruskin | Solutions or tablets for pharmaceutical uses compri ing isotonic solut ons of compounds of an isolated nucleotide such as adenylic ac d and Hg Ca Fe Au Ag or Al |
| B 396 647 | Aug 10 1933 | I G | Alkali metal salt of adenyl pyrophosphoric acid |
| B 413 430 | July 19 1934 | I G | Muscle adenylic acid from muscle adenyl pyrophosphoric ac d by hydrolysis with an alk l ne earth metal hydroxide |
| B 426 8 ʗ<br>G 638 418 | April 10 193›<br>Nov 14 1936 | Ciba | Nucleot des *e g* ad nylic acid are acylated |
| G 591 926 | Jan 29 1934 | C L Lautenschläger and F Lindner<br>As to I G | Isolation of adeno ne phosphoric ac d by hydrolys s of yeast extracts containing adenosine triphosphoric acid |

NICOTINIC ACID

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 403 117<br>B 184 6 5<br>G 351 085 | Jan 10 192<br>Sept 14 1922<br>April 3 19 2 | M Hartman and M Seiberth<br>Ass to C ba | Product claims for dialkyl amides as med c nals Process cla ms reaction of nicot nic acid hal de and a salt of a dialkylam ne |
| U S 1 611 978 | Dec 28 1926 | R Wolffenst n | Terpene alcohol esters of n cot n c acid |
| U S 1 617 332<br>G 441 707 | Feb 15 19 7<br>Mar 10 1927 | M Hartmann and M Seiberth<br>A s to Ciba | Manufacture of pyrid ne-3-carboxylic acid amides substituted in the amide group by reaction of a qu nolinic acid anhydride with a secondary amine followed by decarboxylation |
| U S 1 938 ‘53 | Dec 5 1933 | M Hartmann and M Seiberth<br>Ass to C ba | Add t on product of n cotinic acid and its derivatives with water soluble salts of alkal ne earth metals |
| U S 2 136 501 | Nov 15 1938 | M Hartmann and L Panizzon<br>Ass to C ba | Mercuric N propyl nicotin amides |
| U S 2 136 503<br>Div n of<br>U S 2 136 501 | Nov 15 1938 | M Hartmann and L Pan z zon<br>Ass to Ciba | N substituted n cotinamide mercuric compounds |
| U S 2 141 128 | Dec 20 1938 | H v Euler H Albers and F Schlenk<br>Ass to E B lh ber | Purification of codehydrogenase I by precip t tion with barium hydroxide and fraction l adsorption on alum na Also by prec p tation w th a monovalent copper halide followed by precip tation with barium hydro de |
| U S 2 146 809 | Feb 14 1939 | P Karrer<br>Ass to Hoffmann LaRoche | Manuf cture of 3-carbo ylic acid amides of acetylated N glycosido pyrid nium bromide |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 188 244<br>B 503 780<br>F 824 042<br>F 824 042/49 165 (1st add)<br>F 824 042/49 840 (2nd add) | Jan 23 1940<br>April 12 1939<br>Jan 31 1938<br>Nov 28 1938<br><br>Aug 8 1939 | G A *Langlois* and A M De loison<br>Ass to Fabriques de Produits de Chimie Organique de Loire | Morpholine amide of nicotinic acid |
| U S 2 233 419 | Mar 4 1941 | E E Moore<br>Ass to Abbott | Composition of matter claims for straight chain aliphatic amine salt of nicotinic acid for parenteral in jection |
| B 254 747 | Dec 20 1914 | Ciba | Dialkylamides of nicotinic acid |
| B *450 051*<br>C 653 873<br>F 791 783 | *July 9 1936*<br>Nov 18 1937<br>Dec 17 1935 | K Fricker | Synthesis of N substituted amides of pyridine-carboxylic acids by con densation of the acid with second ary aliphatic or cyclic amines in the presence of phosphorous pentoxide |
| G *529 319* | *July 11 1931* | *Knoll A G* | Purification of cozymase by pre cipitation with picric acid followed by decomposition with silicotung stic acid |
| G 544 389 | Mar 12 1931 | Ciba | Double compound of nicotinic acid with salts such as $CaCl_2$ $MgCl_2$ etc |
| G 554 008<br>Continuation of<br>G 529 319 | July 4 1932 | Knoll A G | Purification of cozymase by precipi tation with aluminum sulfate and *decomposition by means of phos* phoric acid The cozymase ob tained is further purified according to G 529 319 |
| G 603 733 | Oct 6 1934 | Ciba | Urethanes of nicotinic acid |
| G 654 960 | Jan 8 1938 | O Warburg and W Christian<br>Ass to Schering | Isolation of codehydrogenase II |
| G 667 696<br>G 668 388 | Mar 14 1939<br>Mar 22 1939 | H Albert and F Schlenk<br>Ass to I G | Cozymase |
| F 849 519 | Nov 25 1939 | Ass to G Henning | Enzymatic synthesis of muscle adenylic acid and of cozymase from adenosine |
| Hung 110 083 | June 1 1934 | Chinoin Gyógyszer es Veg yeszeti | Free nicotinic acid is heated with substituted carbamyl *chlorides* |
| Russ 35 836 | April 30 1934 | I L Knunyantz and M M Katznel son | Dialkyl amino ethyl esters of nico tinic acid |
| Russ 44 5?4 | Oct 31 1935 | Y L Goldfarb | Preparation of 2 naphthyl nicotin ate |

PANTOTHENIC ACID

| | | | |
|---|---|---|---|
| U S 2 234 680 | Mar 11 1941 | M B Moore<br>Ass to Abbott | Synthesis of pantothenic acid from beta alanine and alpha gamma *dihydroxy beta beta* dimethyl *n* butyric acid or alpha hydroxy beta beta dimethyl gamma butyro lactone |
| U S 2 271 872 | Feb 3 1942 | H K Mitchell<br>Ass to Research Corp | Production of pantothenic acid analogues having in the beta posi tion of the butyric acid part one or two methylol groups |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| B 535 988 | April 29 1941 | Merck | Synthesis of pantothenic acid by condensation of beta alanine or a salt or ester thereof with alpha hydroxy beta beta dimethyl gamma butyrolactone or the corresponding hydroxy or keto-acid |

INOSITOL

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 072 989<br>(Also Swiss<br>62 459 | Sept 9 1913<br><br>June 5 1912 | E Preiswerk<br><br>Ass to Ciba) | Iron salts of inositol phosphoric acid absorbed on albumin |
| U S 1 721 214<br>B 308 403 | July 16 1929<br>Jan 24 1928 | F Goedeck | Dietary composition containing vitamins and inositol he aphosphate and its Ca salts from the same plant source |
| U S 2 112 553 | Mar 29 1938 | E Bartow and W W Walker | Ino tol from phytin by decomposition with $Ca(OH)_2$ |
| B 216 982 | Mar 28 1923 | G Bruni | Methoxyino ityl pentaphosphate |
| G 684 046 | Dec 8 1939 | F Fischler | Isolation of alkaline earth salts of inos tol tetraphosphate |
| Swiss 91 727 | Nov 16 1921 | S Pasternak | Inos tolpolypho phate by phosphorylation of inositol |
| Swiss 141,_2 | Dec 8 1928 | Cristallo A G | Extraction of Ca inos tol he aphosphate and vitam ns from vegetable matter |

VITAMIN C

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Isolation | | | |
| U S 1 468 231<br>B 193 831<br>G 417 020<br>F<br>Add 26 034<br>and 27 271<br>to F 532 398 | Sept 18 1923<br>May 6 1924<br>Aug 8 1925 | L A Agopian | Extract on of vitam ns from fresh plant ju ces to which organ c acids have been added |
| U S 1 745 788 | Feb 4 1930 | C Funk | Antiscorbut c vitamin from lemon ju ce by precipitation as lead salt |
| U S 078,237 | April 27 1937 | O Dalmer and H Wieters Ass to Merck | Isol tion of ascorbic acid from gl d ola leaves |
| U S 2 233 417 | Mar 4 1941 | C G King and W A Waugh | Isolation of vitam n C from lemon ju ce |
| B 133 183 | Oct 9 1919 | Ciba | Vitamin C preparations from vegetable mater als by adding acids as stabilizers and concentrating the mixture |
| B 168 903<br>B 268 655<br>G 415,313<br>F 53 398 | Mar 6 1923<br>April 7 1927<br>June 19 1935<br>Feb 2 1922 | L. A. Agop an | Preparation of vitamins from fresh plant juices by precipitation with salts of heavy metals and by evaporation in the absence of air Extraction of the vitamins with suitabl solvents. |
| B 272 376<br>F 595 537 | June 16 1927<br>Oct 5 19 5 | L A Agop an | Pure vitamin C from plant juices by precip tat on with lead salts and recrystallization from alcohol and acetone |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Isolation** (*Continued*) | | | |
| G 397 886 | June 27 1924 | G Eichelbaum | Antiscorbutic calcium preparation from fruit or plant juices by the addition of calcium hydroxide |
| G 637 258 | Oct 27 1936 | K L Lautenschläger and F Lindner<br>Ass to I G | Isolation of ascorbic acid from leaves of plants of the *Polygonaceae* family |
| G 661 176 | June 14 1938 | K L Lautenschläger and F Lindner<br>Ass to I G | Improved method of isolating ascorbic acid by soaking plant material in solutions of strong acids and further processing according to known methods |
| F 850 539 | Dec 19 1939 | Établissements Byla | Vitamin C concentrates are purified by dialysis in acid solution |
| F 857 981 | Mar 7 1940 | Établissements Byla | Vitamin C extraction from agaves |
| Hung 108 184 | Feb 1 1934 | Ass to Chinoin Gyógyszer | Purification of vitamin C-containing aqueous plant extracts by precipitation with lead and barium salts |
| Russ 48 318 | Aug 31 1936 | A A Schmidt and K S Tutschinskaja | Concentrate of ascorbic acid from hips |
| **Synthesis** | | | |
| U S 2 056 126<br>B 425 198<br>G 624 509<br>F 770 816 | Sept 29 1936<br>Mar 8 1935<br>Feb 11 1936<br>Sept 21 1934 | T Reichstein<br>Ass to Hoffmann LaRoche | *l* Ascorbic acid from *l* xylosone by condensation with hydrocyanic acid followed by hydrolysis |
| U S 2 068 453<br>*B 460 586*<br>G 637 448 | Jan 19 1937<br>*Feb 1 1937*<br>Oct 29 1936 | B Helferich and O Peters | Vitamin C by condensation of glyoxylic acid ester with *l* threose |
| U S 2 073 707<br>B 443 901<br>F 794 221 | Mar 9 1937<br>Mar 2 1936<br>Feb 11 1936 | W N Haworth E L Hirst J K N Jones and F Smith<br>Ass to British Drug Houses | Ascorbic acid is prepared by oxidizing *l* sorbose with nitric acid to 2 keto *l* gulonic acid and enolizing |
| *U S 2 129 317* | *Sept 6 1938* | F Elger<br>Ass to Hoffman LaRoche | Ascorbic acid by acid treatment of 2 keto *l* gulonic acid its ester or diacetone compound |
| U S 2 159 191<br>B 461 790<br>G 641 639<br>*F 606 976* | May 23 1939<br>Feb 24 1937<br>Feb 13 1937<br>*Dec 29 1936* | W Wenner<br>Ass to Hoffmann LaRoche | Ascorbic acid from bis methylene ethers of 2 keto *l* gulonic acid or diacetone 2 keto-*l* gulonic acid by means of acids at elevated temperatures |
| U S 2 160 621<br>B 430 264<br>G 648 311 | May 30 1939<br>June 17 1935<br>Aug 20 1937 | H Ohle | Ascorbic acid from 2 keto gulonic acid by basic agents |
| *U S 2 165 151*<br>B 521 831 | *July 4 1939*<br>May 31 1940 | R Pasternack and P P Regna<br>Ass to Pfizer | *l* Ascorbic acid from esters of 2 keto *l* gulonic acid by treatment with a metal *e g* iron nickel cobalt manganese cadmium and zinc in aqueous solution |
| U S 2 165 184 | July 4 1939 | R Pasternack and P P Regna<br>Ass to Pfizer | *l* Ascorbic acid from esters of 2 keto *l* gulonic acid by treatment with magnesium in water and alcohol or dioxane solution |
| U S 2 179 977 | Nov 14 1939 | F Elger<br>Ass to Hoffmann LaRoche | *l Ascorbic acid from 2 keto-l* gulonic acid or derivatives thereof by acid treatment in a mixture of alcohol and chloroform |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Synthesis (*Continued*) | | | |
| U S 2 179 978<br>G 104 760<br>G 673 485 | Nov 14 1939<br>April 7 1941<br>Mar 2 1939 | F Elger<br>Ass to Hoffmann LaRoche | *l* Ascorb c acid from 2 keto-*l* gulonic acid or its alkyl esters by heat ng in the presence of an alkal salt of a weak acid in anhydrous alcohol |
| U S 2 18 ,383<br>B 516 115 | Jan 2 1940<br>Dec 22 1939 | R Pasternack and G O Cragwall<br>Ass to Pfizer | *l* Ascorbic ac d from 2 keto *l* gulonic acid by the action of glacial acetic acid and a mineral ac d |
| U S 2 189 830<br>G 676 011 | Feb 13 1940<br>May "4 1939 | O Z ma<br>Ass to Merck | *l* Ascorbic acid from diacetone 2 keto gulon c acid by ac d treatment |
| U S 2 189 778 | Feb 13 1940 | O Dalmer and K Heyns<br>Ass to Merck | *l* Ascorbic acid from sorbose by catalyt c oxidation followed by ac d treatment |
| U S 2 190 167<br>G 684 72 | Feb 13 1940<br>Dec 4 1939 | O Zima<br>Ass to Merck | *l* Ascorbic acid from methylene ether derivatives or dibenzal de rivatives of keto *l* gulonic acid by treatment with concentrated HCl |
| U S 2 20^ 374 | July ^ 1940 | I Stone<br>Ass to Wallerste n Co | Ascorbic acid from osones plus hydrocyanic ac d the osone being p epared by oxid tion of sug rs with cupr c ion in aqueous organic acid solut on |
| U S 207 080<br>( 683 9 4 | July 9 1940<br>Nov 18 1939 | B Helferich<br>Ass to Winthrop | Asco b c ac d by condensat on of mesoxal c acid ester with *l* threose |
| U S 2 1 1 | Dec 9 1941 | T Reichst n<br>Ass to Hoffmann LaRoche | Ascorbic ac d from *l* sorbose *v a* 2 keto-*l* gulon c ac d or methyl e ether derivative thereof |
| B 4^8 814<br>C 673 485<br>F 779 883 | May 20 1935<br>Mar 28 1939<br>April 13 1935 | T Re chste n<br>Ass to Hoffmann L Roche | Ascorb c cid from 2 keto-*l* gulonic acid sters by alk l saponification followed by lacton zat on n acid sol tion |
| B 4 8 815 | M y 20 1935 | T Re chste n | Ascorb c acid from ^ keto-*l* gulonic acid by acid treatment |
| B 4 9,207 | Jan 4 1937 | T Reichstein | Ascorb c aci l from 2 keto *l* gulo c acid or its de ivatives wh ch re ea ly split with ac ds by ac ds in alcohol c solut on |
| B 40( 48 | May 31 1937 | T Re chste n | Ascorbic ac d by heating 2 keto gulonic ac d or its derivat ves wh ch are easily spl t with acids |
| B 469 1 7 | July 20 1937 | T Reichste n | Ascorbic acid from ^ keto gulon c acid by treating w th alkali salts of weak acids and converting the so-formed alkali salts of ascorb c acid by means of strong acids into ascorbic acid |
| ( 07 0 4<br>Cont lat n of<br>( 6 3 48 | June 1 1941 | Hoffmann LaRoch | Ascorb c acid from 2 keto-*l* gulonic acid or deri t ves wh ch are hydrolyzed easily by acids by tre tment w th ac ds in the presence of a sol ve t n which the formed ascorb c ac d is insoluble |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| *Intermediates for the Synthesis* | | | |
| U S 2 140 480<br>U S 2 039 929<br>B 428 815<br>B 4?7 ?86<br>B 435 971<br>G 699 877<br>G 703 2?7<br>F 780 0?5<br>F 45 774 | Dec 13 1938<br>May 6 1936<br>May 20 1935<br>April 18 1935<br>Oct 2 1935<br>Nov 7 1940<br>Mar 4 1941<br>April 18 1935<br>Dec 3 1935 | T Reichstein<br>Ass to Hoffmann LaRoche | 2 Keto *l* gulonic acid from sorbose *via* a bis methylene ether derivative or acetals of cyclic ketones by oxidation with permanganate |
| U S 2 153 311 | April 4 1939 | R Pasternack and P P Regna<br>Ass to Pfizer | Oxidation of *l* gulonic acid to 2 keto *l* gulonic acid by chromic acid |
| U S 2 157 137 | April 9 1939 | R T Major and E W Cook<br>Ass to Merck | Xyloseen tribenzoates and proces for producing the same |
| U S 2 188 777<br>B 534 746 | Jan 30 1940<br>Mar 17 1941 | R Pasternack and P P Regna<br>Ass to Pfizer | Oxidation of soluble aldonates to 2 keto aldonates by means of chlorates in the presence of a vanadium catalyst |
| U S 2 190 377<br>B 495 050<br>F 829 236 | Feb 13 1940<br>Nov 7 1938<br>June 16 1938 | O Dalmer and K Heyns<br>Ass to Merck | 2 Keto *l* gulonic acid from sorbose by catalytic oxidation at pH of about 6 to 11 |
| U S 2 194 476<br>See<br>U S 2 198 628 | Mar 26 1940<br><br>April 30 1940 | R T Major and F W Cook<br>Ass to Merck | Nitriles of fully acetylated 2 keto sugar acids and process for their production |
| U S 2 198 628 | April 30 1940 | R T Major and F W Cook<br>Ass to Merck | Fully acetylated sugar acid chlorides<br>*To be used in U S 2 194 476* |
| U S 2 207 991 | July 16 1940 | R Pasternack and P P Regna<br>Ass to Pfizer | 2 Keto aldonic acid esters from aldonic acids by chlorates in the presence of a vanadium catalyst and a mineral acid using non aqueous lower alcohols as solvents |
| U S 2 2?2 155 | Nov 11 1940 | R Pasternack and P P Regna<br>Ass to Pfizer | 2 Keto aldonic acids by subjecting an aldonate to anodic oxidation in a weakly acid aqueous solution containing a soluble chromium compound and a member selected from the group consisting of alkali metal and alkaline earth metal chlorides and bromides |
| G 544 666 | Feb ?0 193? | J Muller and U Hoffman<br>Ass to I G | Manufacture of polyvalent alcohols by catalytic reduction of sugars with hydrogen |
| G 627 24? | Mar 23 1936 | Hoffmann LaRoche | *l* Xylose from *d* sorbitol acetate by oxidation |
| G 644 ?6? | May 19 1937 | Hoffmann LaRoche | 2 Keto-*l* gulonic acid from *l* sorbose by direct oxidation with $H_2O$ |
| I 851 347 | Jan 6 1940 | A Corbellini | The acetone condensation product of a 2 keto laevo gulonic acid is heated with methanol and sulfuric acid to form methyl ethers with the hydroxyl groups of the acid These methyl ethers are of value in the synthesis of laevo-ascorbic acid (vitamin C) |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Intermediates for the Synthesis** (*Continued*) | | | |
| Swiss 175 347 | May 1 1935 | T Re chstein | Preparation of the methyl ester of keto-hexon c acid from the free acid with HCl or $H_2SO_4$ in methanol |
| **Derivatives and Utilization** | | | |
| U S 1 886 931 | Nov 2 1932 | E R Alexander<br>Ass to the V tamin Corp | Process of preserving a concentrated vitamin e tract by incorporation into a vitamin preserving acidic fru t ma s |
| U S ? 035 153<br>B 439 93,<br>G 660 "03 | Mar 24 1936<br>Dec 17 1935<br>June 1 1938 | F Elg r<br>A s to Hoffmann LaRoche | Ascorb c acid as stab lizer for neo salvarsan |
| U S 2 058 2?0<br>G 6 9 7 3<br>F 788 014 | Oct 20 1936<br>May 9 1936<br>Oct 10 193 | 1 Elger<br>A s to Hoffmann LaRoche | Addition of ? keto-*l* gulonic acid esters to foods |
| U S 2 117 777<br>G 70 185 | May 17 1938<br>Feb 4 1941 | K Warnat<br>Ass to Hoffmann LaRoche | Double salt of calcium ascorbate and of cal ium salts of poly hydroxy mono-carbo yl c ac ds |
| U S 2 132 66 | Oct 11 1938 | I H Volwiler and M B Moore<br>Ass to Abbott | Al phatic amine salts of ascorbic a id and proce s for producing them |
| U S 134 ?46<br>B 480 503 | Oct 25 1938<br>Feb 23 1938 | F Liger<br>Ass to Hoffmann LaRoche | Ascorb c ac d salts of h stid ne |
| U S 2 140 989<br>B 499 798<br>B 499 840 | Dec 20 1938<br>Jan 30 1939<br>Jan 30 1939 | J Eisenbrand and M L enz<br>Ass to Winthrop | Molecular compounds from ascorbic ac d or iso a corb c acid and qu nine or quin d ne |
| U S 2 150 140 | Mar 7 1939 | K Warnat<br>Ass to Hoffmann LaRoche | 3 o 6 Tribenzoyl ascorb c acid and its preparation from ascorbic acid salts and benzoyl chloride |
| *U S ? 159 214* | *May 23 1939* | S Kle n | *Double salt of calcium* asco bate and calc um cetylsalicylate |
| U S 2 159 986 | May 30 1939 | P P Gray a d I St ne<br>Ass to Wall rstein | Addit on of ascorb c acid to aque ous o l emul ons |
| U S 2 187 467 | Jan 16 1940 | E H Stuart<br>Ass to El Lilly | Aqu ous solution of a salt of ascor bic acid *e g* of alkal metal alk li e earth metal ammonium the lower alkyl s b tit ted am nes and contain ng s lfur compounds *e g* sodium hydro ulf t |
| U S 212 831 | Aug 27 1940 | F Hoffm n and P M rquardt<br>A s to Byk Guldenwerke | Manufacture of stable derivat ves of adrenal ne by reacting adrenal ne w th ascorbic acid |
| U S 2 213 977 | Sept 10 1940 | W G Chr stiansen<br>Ass to Sq ibb | Vitam n C am n solut on stabil zed by mean of hypophosphite |
| U S 2 3? ?99 | Feb ? 1941 | U H Engels J We jlard and R T Schenck<br>Ass to Merck | Stab l zation of asc rb c acid solu t ons by water soluble ed ble col lo ds of the group cons sting of albumen ac cia case n raw m lk gelat n and starch |
| U S 2 ?32 712 | Feb ?5 1941 | R T Major nd E W Cook<br>Ass to Merck | Process for the prod ct on of f lly acetyl ted sugar acids from corre spond ng aldehydo-sugar acetates and corre pond ng 4 lactones Composit on of m tter claims for f lly acetylated sugar acids wh ch do not cont in a keto group |

| Patent No | Date | Patenter | Abstract |
|---|---|---|---|
| **Derivatives and Utilization** (*Continued*) | | | |
| U S 2 249 903<br>G 693 375 | July 22 1941<br>July 6 1940 | C L Lautenschläger and F Lindner<br>Ass to I G | Preparation of stable water solutions of ascorbic acid by partial or complete neutralization with organic bases |
| U S 2 2₀1 526<br>B 524 319 | Aug 5 1941<br>Aug 2 1940 | A Salomon<br>Ass to N V Orgachemia | Stable concentrated quinine solutions comprising dissolving a mono salt of quinine with the acid of ascorbic acid |
| U S 2 2 5 483 | Sept 9 1941 | G F D Alelio<br>Ass to General Electric | Addition of ascorb c acid as an inhibitor to polymerizable materials containing a methylene group |
| U S 2 260 870 | Oct 28 1941 | S I Ruskin | Manganese compoun l of a corbic acid |
| B 45₀ 921<br>F 79? 675 | Oct 28 1936<br>July 1 1936 | Dansk Gaering Industri | Addition of ascorbic aci l to flour and dough |
| B 469 33₀ | July 23 1937 | I G | Stable solutions of partly or completely neutralized ascorbic acid for ampules |
| B 472 ₀31<br>F 817 578 | Sept 20 1937<br>Sept 6 1937 | Ciba | Process for the manufacture of ferrous salts of ascorbic acid |
| B 48₀ 61?<br>F 82) 982 | May 23 1938<br>July 18 1938 | H Lotze | Addition of catalase to preparations containing vitamins A B C and/or D for protecting vitamin C against destruction |
| B 486 05₀ | May 20 1938 | H W Rhodehamel and E C Kleiderer<br>Ass to Eli Lilly | Anhydrous solution of vitamin C in propand ol |
| B 486 546 | June 7 1938 | P K Henriksen<br>Ass to Canned Cream & Milk Co | Addition of vitamin C to milk or l qu d milk products |
| B 486 7₀7<br>G 68? 875 | June 9 1938<br>Oct 24 1939 | F B Dehn<br>Ass to Promonta | Preparation of ferrous salts of ascorbic acid |
| B 488 784 | July 13 1938 | S L Ruskin | Process for the product on of the follow ng salts of a corb c acid alkali calcium barium trontium iron manganese bi muth arsenic gold s lver copper merc ry zinc alum num and tin |
| *B 495 675* | *Nov 17 1938* | *Hoffmann LaRoche* | *Process for the manufacture of cal*cium double salts of ascorbic acid and poly oxy mono carboxylic acids |
| B ₀03 476 | Mar 30 1939 | N V Industrielle Maatschappij vorheen Noutry & van der Lande | M xture of ascorbic acid with calcium phosphates gypsum silica talc, magnesium carbonate potass um sulfate wheat rice maize or potato starch |
| B ₀09 709 | July 19 1939 | N V Orgachemia | Stabilizat on of ergometrine by ascorbic acid |
| B ₀11 585 | Aug 21 1939 | Ocean | Addition of vitamin C cont in ng paprika to foodst ffs |
| B ₀14 047 | Oct 30 1939 | L E Wells and Rountree Co | Particles of ascorbic aci l coated with gelat ne gum or h rd sug r |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Derivatives and Utilization (Continued) | | | |
| B 517 348<br>F 829 547 | Jan 26 1940<br>June 29 1938 | P R A Maltha | Addit on of ascorbic acid to form in a mixture with metals or salts of copper manganese cobalt iron salts prote c acid salts or proteins containing such metals or powder of squash cucumbers cabbage leaves or tomatoes |
| B 533 480 | Feb 13 1941 | Hoffmann LaRoche | Ascorbic ac d salts of cinchona alkaloids |
| G 470 01? | Jan 3 19?9 | J Korselt | Preparation of pressed plant juices rich in v tamins from chlorophyll contain ng plants by the addition of calcium salts and al phat c oxy acids |
| G 639 776<br>(See cont nua tion 701 561) | Dec 1? 1936 | Hoffmann LaRoche | Preparat on of esters of ascorb c acid and higher fatty acids |
| G 663 987 | Aug 18 1938 | Merck | Solut on of mono alkali or alkaline earth salts of ascorbic acid for filling amp iles followed by sterilization |
| G 681 859 | Oct 3 1939 | Madaus & Co | Preparation of stable colloidal solu tions of phosphorus n alcohol by the add tion of ascorb c acid |
| G 696 794 | Sept 30 1940 | Madaus & Co | Stab l zation of collo dal bismuth contain ng aqueous solutions for in jections by add ng ascorb c acid in the presence of protective colloids |
| G 699 327<br>Continuation of<br>G 693 375 | Nov 7 1940 | C L Lautenschläger and F Lindner<br>Ass to I G | Preparation of stable water solu tions of ascorbic ac d by p rtial neu tral zation w th inorgan c bases |
| G 701 561<br>Continuat on of<br>G 639 776 | Jan 18 1941 | Hoffmann LaRoche | Esters of ascorb c ac d by reaction of salts of ascorb c acid with ac d hal des of aromat c carboxyl c acids or by re ction of ascorb c acid w th ac d hal des in the presence of organ c ba es |
| F 749 74 | July 28 1933 | C Croll n I I St rnmann | Add t on of steril zed plants con taining vitam n C to evaporated m lk |
| F 800 50? | July 8 1936 | Soc Fr nçai e D Sucres | Add t on of solut ons of vitamins C and D to sugar or sugar syrup |
| F 805 335 | Nov 17 1936 | P Arlo ng A Morel and A Jo serand | Prep ration of complex salts of a co b c acid |
| F<br>805 335/47 1?5 | Feb 6 1937 | I Arloi g A Mor l and A Josserand | Pr parat on of ammonium or am ne-conta n ng complex salts of ascorb c acid |
| F 807 877 | Jan 23 1937 | I Arlo ng A Morel and A Jo erand | Prep ration of comple salt of asc rb c aci l |
| I 8 3 73? | J n ?? 1938 | I Arlo ng A Mo el and A Jos erand | P eparat on of compl x salts of iso vitamin C. |
| F 8?7 14? | April 20 1938 | N V Ind istr elle Maatsch p j vorheen Noutry & van der Land | Preservation of food by the add t on of ascorb acid together w th non hygroscopic subst nce |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Provitamins D** (*Continued*) | | | |
| B 504 051 | April 19 1939 | The International Yeast Co and W G Bennett | Extraction of ergosterol from yeast by plasmolysis or boiling with acid followed by extraction with an organic fat solvent and *saponification* |
| G 542 667 | Jan 28 1932 | Merck | Ergosterol from yeast residues |
| G 553 91[illegible] | July 2 1932 | Hoffmann LaRoche | Isolation of sterols from yeast by heating yeast with aqueous alkali solutions in an open vessel |
| Austrian 140 190 | Jan 10 1935 | W Halden | Cultivation of yeast high in ergosterol under aerobic conditions |
| Belg 416 160 | June 19 1936 | N V Philips Gloeilampen fabrieken | Purification of ergosterol by esterification followed by absorption |
| Swiss 129 879 | Jan 2 1939 | Ciba | Isolation of ergosterol from fungi by alkali saponification |
| Swiss 201 169 | Feb 1 1939 | I G | Preparation of 7 dehydro cholesterol from 7 oxo cholesterol by reduction to 7 hydroxy cholesterol diacylation heat decomposition to a 7 dehydro cholesterol ester and final saponification |
| ***Conversion of Provitamins D to Vitamins D*** | | | |
| U S 1 680 818<br>B 236 197<br>F 587 187 | Aug 14 1928<br>June 30 1924<br>April 14 1935 | H Steenbock<br>Ass to Wisconsin Alumni Research Foundation | Antirachitic activation of foods by ultraviolet irradiation |
| U S 1 681 120 | Aug 14 1928 | A J Pacini<br>Ass to M Richter | Production of antirachitic material from growth producing substances by irradiation with light of wave lengths longer than 30[illegible] Å |
| U S 1 682 318 | Aug 28 1928 | F C Beardslee<br>Ass to F C Beardslee B M Huffine and J I Huffine | Apparatus for ultraviolet irradiation of food materials |
| U S 1 704 173 | Mar 5 1929 | J W D Chesney<br>Ass to Solar Research Corp | Method of making the ultraviolet rays of the sun available for the production of the anti rachitic principle in substances susceptible to such activation by separating solar ultraviolet rays of wave lengths within the range of substantially 2900 to 3700 Angström Units from the remaining rays and intensifying *the separated ultraviolet rays* by concentration |
| U S 1 723 603 | Aug 6 1929 | J W D Chesney<br>*Ass to Chesney Process Inc* | Process for activating pasteurized liquids by addition of an organic acid followed by ultraviolet irradiation |
| U S 1 754 434 | April 15 1930 | J Perino | Vegetable alimentary materials are subjected in the absence of oxygen and in the presence of soluble phosphates to an irradiation by ultra violet rays and are heated to not above 60 C during the irradiation |
| U S 1 762 105<br>B 356 793 | June 3 1930<br>June 6 1930 | A J Pacini<br>Ass to C M Richter | Vitamins D by irradiation of sterols or fats with infra red or ultraviolet light in inert gas. |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Conversion of Provitamins D to Vitamins D (*Continued*) | | | |
| U S 1 771 343 | July 22 1930 | A J Pacini<br>Ass to C M Richter | Production of vitamin D by irradiation of sterols in the presence of a suitable photocatalyst such as halogen or by rays longer than 3022 Å or by electromagnetic radiation in the presence of a catalyst |
| U S 1 796 134<br>G 564 401<br>G 608 77<br>G 647 5 2 | Mar 10 1931<br>Nov 18 1932<br>Jan 19 1935<br>July 6 1937 | A Wörner and F Kielwein | Claims a baking oven containing a mercury vapor lamp and a reflector for indirect irradiation and also claims irradiation of yeast therein |
| U S 1 808 760 | June 9 1931 | C E Bills<br>Ass to Mead Johnson | Apparatus for irradiating liquids such as oils mixed with ergosterol |
| U S 1 817 936 | Aug 11 1931 | G C Supplee<br>Ass to The Borden Co | Process of irradiating milk with ultraviolet rays |
| U S 1 842 313 | Jan 19 1932 | N K Chaney<br>Ass to National Carbon Co | Apparatus for irradiating foods with ultraviolet rays |
| U S 1 848 305 | Mar 8 1932 | C E Bills<br>Ass to Mead Johnson | Method of irradiating a stream of fluids |
| U S 1 871 135 | Aug 9 1932 | H Steenbock<br>Ass to Wisconsin Alumni Research Foundation | Preparation of antirachitic food substances by ultraviolet irradiation of cereals |
| U S 1 871 136<br>B 314 94<br>G 60, 960 | Aug 9 1932<br>Aug 28 1929<br>Nov 2? 1934 | H Steenbock<br>Ass to Wisconsin Alumni Research Foundation | Antirachitic product by ultraviolet irradiation of lipoids |
| U S 1 873 942<br>B 293 557 | Aug 23 193?<br>Not accepted | A Windaus<br>(In Great Britain—E Merck) | Antirachitic preparations by irradiation of ergosterol solutions until digitonin yields only a small precipitate |
| U S 1 880 977 | Oct 4 1932 | A J Pacini<br>Ass to Sun A Sured Inc | Production of vitamin D by heating lipoids such as cholesterol with a photocatalyst such as uranium acetate. |
| U S 1 880 978 | Oct 4 1932 | A J Pacini<br>Ass to Sun A Sured Inc | Process of producing vitamin D by heat extracting ergosterol from natural sources in the presence of a photocatalyst |
| U S 1 894 158 | Jan 10 1933 | N K. Chaney<br>Ass to National Carbon Co | Antirachitic activation of food stuffs by irradiation from an arc |
| U S 1 896 191<br>B ?96 093<br>B 316 803<br>G ?499 524<br>F 659 448 | Feb 7 1933<br>Aug ?6 19?7<br>Aug 29 1929<br>June 7 1930<br>Aug 24 1928 | W Zimmermann and W Frankenburger<br>Ass to Winthrop | Production of vitamin D from ergosterol by ultraviolet rays and interrupting the exposure before the maximum absorption in the range of the spectrum between λ = 300mμ and λ = 230mμ has been reached |
| U S 1 904 751<br>B 343 5 8<br>G 634 146<br>F 700 312<br>F 40 142 | April 18 1933<br>Mar 19 1931<br>Aug 18 1936<br>Feb 27 1931<br>Apr 120 1932 | E H Reerink and A. van Wijk<br>Ass to N V Philips Gloeilampenfabrieken | Irradiation of ergosterol with wave lengths from 270 to 300 mμ. |
| U S 1 920 587 | Aug 1 1933 | A. J Pacini<br>Ass. to Sun A-Sured Inc | Method of producing vitamin D comprising suspending lipoids such as cholesterol and treating them with aspergillus oryzae. |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Conversion of Provitamins D to Vitamins D (*Continued*) | | | |
| U S 1 928 397 | Sept 26 1933 | E D Shumway<br>Ass to Quaker Oats | Production of an antirachitically activated cereal by removing the husk or skin from cereal and subjecting the whole kernel to the action of activating rays |
| U S 1 954 065 | April 10 1934 | J H Bragg | Apparatus for increasing the vitamin content of liquid food comprising a food chamber having openings for the passage of liquid food a source of ultraviolet rays and means to effect a cooling of the contents of the chamber |
| U S 1 955 554<br>B 394 408 | April 17 1934<br>June 29 1933 | R F Light and C N Frey<br>Ass to Standard Brands | Process of activating antirachitically activatable unsaponifiable lipoids in dioxane by light containing frequencies below the visible spectrum |
| U S 1 966 546<br>U S 1 966 547 | July 17 1934<br>July 17 1934 | G P Goode<br>Ass to General Development Lab | Apparatus for irradiating solutions of ergosterol etc with filtered ultraviolet light |
| U S 1 980 971 | Nov 13 1934 | H G Campsie | Production of vitamin D from provitamin D by means of radiant energy of 2536 to 2540 Angström Units |
| U S 1 982 028 | Nov 27 1934 | G Sperti<br>Ass to General Development Laboratories | Process of increasing the vitamin content of food substances by subjecting them to soft x rays having wave lengths between approximately 2 and 13 Angström Units |
| U S 1 982 029 | Nov 27 1934 | G Sperti R J Norris R B Withrow and H Schneider<br>Ass to General Development Laboratories | Process for treating food substances with ultraviolet light |
| U S 1 983 944 | Dec 11 1934 | A J Pacini<br>Ass to American Research Products | Process of activating provitamin D by treatment with cathode rays in the presence of a suitable catalyst such as chlorine or bromine |
| U S 2 007 765<br>B 292 926 | July 9 1935<br>Aug 22 1929 | A Knudson<br>Ass to Sun A Sured Inc | Process of increasing the vitamin D content of food fats or ergosterol by treatment with high velocity electrons |
| U S 2 015 264<br>U S 2 015 265 | Sept 24 1935<br>Sept 24 1935 | R M Fraps | Apparatus for irradiating materials such as ergosterol by sunlight |
| U S 2 015 282 | Sept 24 1935 | A J Pacini<br>Ass to American Research Products Inc | Production of vitamin D from provitamin D by radioactive subsubstances |
| U S 2 057 399 | Oct 13 1936 | H Steenbock<br>Ass to Wisconsin Alumni Research Foundation | Yeast is antirachitically activated by treatment with light rich in ultraviolet rays |
| U S 2 104 681 | Jan 4 1938 | G C Supplee<br>Ass to The Borden Co and to National Carbon Co | Method of irradiating liquid milk products with ultraviolet energy to give increased antirachitic potency by impinging the ultraviolet energy in such a manner that all rays of energy impinge obliquely upon the surface |

| Patent No | Date | Patentee | Abstract |
| --- | --- | --- | --- |
| Conversion of Provitamins D to Vitamins D (*Cont'd*) | | | |
| U S 2 106 773 | Feb 1 1938 | C C Whittier | Product on of vit m 1 D l y pas ing a vaporized provitamin D through a zone of electrically induced anti rachitically activating discharge in a vacuum containing radium ema nations |
| U S 2 106 780 | Feb 1 1938 | C C Whittier | Method of producing vitamin D which consists in vaporizing ergo-sterol passing the same through a zone of electrical discharge in a vacuum tube condensing the treated ergosterol vapor and sub jecting the condensate to a trans versely directed electrical d scharge in the acuum tube |
| U S 2 106 781 | Feb 1 1938 | C C Wh ttier Ass to Nutrition Research Laboratories | Apparatus for the activation of pro-vitamin D by the electrical dis-charge method |
| U S 2 106 782 | Feb 1 1938 | C C Wh ttier Ass to Nutr tion Re earch Laboratories | Apparatus for the activat on of pro-vitamin D by the electr cal dis charge method |
| U S 2 11 242 | Mar 29 1938 | B Kramer and A E Sob l | Process for the ant rach tic activa-tion of provitamins D by an electr cal d scharge method |
| U S 2 117 100 | May 10 1938 | N A Mil s Ass to DuPont | Preparation of antirachitic sub stances from prov tamins D by the action of a high frequency electri l osc llating discharge |
| U S 2 128 199<br>G 642 759 | Aug 23 1938<br>Mar 16 1937 | A Windaus Ass to Winthrop | Pr par tion of an antirach tic lly active substance by ultra olet ir radiat on of 2 3-dihydro ergo sterol |
| U S 2 151 645 | Mar 21 1939 | H C Stephens an l S B Hoar Ass to Natural Food Prod ucts | Method for de erating a l qu d food product in vacuum and while under vacuum e po ing the c m pletely deaerat d liqui l to ultra violet irrad ation |
| U S 2 183 933 | Dec 19 1939 | J K Elderkin and E Holman Ass to Chemi al Products Co | Foods are antirach t cally act ated by the action of ozone |
| U S 2 202 611 | May 28 1940 | G C Supplee and J Dorcas Ass to The Borden Co and National Carbon Co | Method and apparatu f r irradia tion of m lk in turbulent flow to in crease the v tam n D content |
| U S 2 31 870 | Feb 18 1941 | W Baeckler Ass to Union Carb de & Car bon | Apparatus for open air irradi tion of l quids such as m lk by flow ng the liquid in a s bstant ally rect l near d rect on along a smooth sur face |
| U S 2,231 871 | Feb 18 1941 | W Baeckler Ass to Un on Carb de & C r bon | Apparatus for open a r irrad ation of l quids such as m lk by us g a con cal type of support for th l qu d in order to maintain a con tant rate of flow |
| U S. 234 554 | Mar 11 1941 | H W Liley and J Waddell Ass to DuPont | The process wh ch comprise dis solving a prov tam n D-containing m terial in an organ c solvent dd ing a sugar amine expo ng the solut on to ultrav olet l ght and re co ering a vitamin D conc ntr te |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| *Conversion of Provitamins D to Vitamins D (Continued)* | | | |
| U S 2 243 632 | May 27 1941 | M L Johnson<br>Ass to Vitamin Technologists | Production of vitamin $D_2$ from ergo terol by irradiation with ultra violet light of 2536–2540 Angström Units |
| U S 2 260 823 | Oct 28 1941 | E S Bettis<br>Ass to Pet Milk Co | Irradiation of milk in a thin film |
| B 265 910 | April 7 1927 | K. Hoefelmayr | Milk in concentrated form or in a curdled state is subjected to the action of artificially produced violet rays by which it is converted into an invalid food effective for the cure of certain diseases |
| B 266 101 | Feb 24 1927 | O A Elias | Method of manufacturing b scuits bread cakes and similar food prod ucts characterized by carrying out the baking process in the presence of artificially produced ultraviolet rays |
| B 270 296 | April 28 1926 | H C E Tillisch | Antirachitic v tamin by ultraviolet irradiation of oils or fats |
| B 285 083 | Jan 24 1929 | Merck | Manufacture of antirachitic prep arat ons by esterifying the un saponifiable constituents of yeast fat or of the corresponding extracts from ergot or similar lower fungi and exposing the esters to the action of an activating radiation or by first activating and afterwards esterifying the material |
| B 286 665 | Sept 6 1928 | Merck | Manufacture of antirachitic sub stances by irradiation of provita mins D in the presence of photo chemical sensibilisers such as eosin or iodine |
| B 290 195 | Mar 7 1929 | C. Jaeger | Ultraviolet irradiation of dried bananas to produce an antirachitic food |
| B 293 255 | Nov 28 1927 | T D Kelly | Oils f t or emulsions are treated with beta rays and with ultra v olet rays to produce vitamins |
| B 295 757 | Aug 23 1928 | I S MacLean | Yeast is incubated in a solut on con taining phosphates and carbohy drates and the sterols or sterols and fats obta ned are subjected to acti vating radiation |
| B 296 053 | Aug 24 1927 | A J Pacini<br>*Ass to M Richter* | Process of treating materials to *form antirachitic* substances by various rays other than ultraviolet rays *e g* by x rays canal rays cathode rays etc |
| B 298 585 | Dec 5 1928 | Dry Milk Co | Ultraviolet irradiat on of milk |
| B 302 980 | Sept 24 1927 | N Bendixen | Rotary device for tre ting liquids with rays or emanations |
| B 309 601<br>F 672 318 | April 13 1928<br>Mar 29 1929 | E Oppenheim | Ultrav olet irradiat on of chocolate |

| Patent No | | Date | Patentee | Abstract |
|---|---|---|---|---|
| Conversion of Provitamins D to Vitamins D (*Conti ued*) | | | | |
| B<br>G | 313 558<br>5.4 874 | June 14 1928<br>Sept 13 19 8 | G Zecher<br>Ass to N V Ph lip Gloei lampenfabrieken | *Apparatus for irrad at ng sub*stances w th ultraviolet l ght |
| B | 314 267 | Aug 20 1928 | I G | Apparatus for the irrad at on of *ergo terol in circulat ng olvent* liquids |
| B | 316 264 | Sept 18 1929 | O R ed | Treatme t of *foo ls* and *f ts* w th short waves ultrav olet rays or x r ys |
| B | 318 268 | S pt 2 1929 | I M H lbron | Act v t on of prov tam n D by *metall c cataly ts at ele ated tem*perature |
| B | 318 269 | Sept 2 1929 | I G | Production of antirach t c products by treat ng ergo terol or substances *containing ergo terol w th corona* d scharges or with electr c corpuscular rays or Roentgen rays while x clud ng oxygen |
| B<br>F | 321 992<br>675 558 | Nov 25 1929<br>May 10 1929 | I G | *Prod ction of v tam n D by irrad a*t on of sterols n the presence of substances wh ch exert a protective action on the v tam n formed such as ether olefin c hydrocarbons or caustic alkal es |
| B. | 324 503 | Mar 19 1930 | Patent Treuhand Ges f elektr Glühlampen | Apparatus for ultraviolet irrad a tion of milk |
| B | 325 470 | July 12 19 9 | J O Hickman and N V Hickman | In a process for the irradiation of flowing m lk in th n layers the n tens ty of irradiat on and rate of flow of the m lk a e regulated |
| B<br>F | 342 500<br>697,367 | Feb 15 1929<br>Jan 30 1930 | T Reiter | V tamin D by irrad ation of ergosterol with light of wave lengths about 280 mμ |
| B | 346 682 | Oct 15 1929 | V C From C D Rowley and A W Lar ky | Simultaneous infra red and ultra violet light irrad ation of m lk |
| B<br>F | 357 2 3 | Jan 4 1930 | F F Tisdall | Wheat germ whole wheat and yeast treated w th ultraviolet light are used in the manufactur of bread cakes etc |
| B<br>F | 38.6 6<br>714 827 | Dec .0 1932<br>April 4 1931 | N V Ph lip Gloeilampen fabrieken | Irrad ation of ergosterol with ultra iolet light us ng a filter which ab orbs light of wave lengths 312 313 mμ e g C$l_2$ |
| B | 403 650 | Jan 10 1934 | J Waerham | Vitam n D conta n ng soap by ir rad at on of soap containing ergo terol or of o ls conta ni g ergosterol followe l by sapon fication |
| B | 489 14 | July 20 1938 | H F Rost | Ultraviolet irrad t on of thin films of pro tam ns D in sol t on o susp n on n the presence of a r with r ys of wave length betw en 2600 and 3000 Angström Un ts |
| B | 497,16 | Dec 14 1938 | N trit on R arch Laboratories | Ant rach t c act at on of pro ita m n D l y sub ecting the por to a lent non lum ou flow of electr c ity |

| Patent No | | Date | Patentee | Abstract |
|---|---|---|---|---|
| **Conversion of Provitamins D to Vitamins D** (*Continued*) | | | | |
| B | 498 068 | Jan 3 1939 | H F Glunz | Process for the irradiation of liquids and the sterile filling of containers therewith in which a zone of ultra violet rays is maintained within a vacuum chamber to irradiate the liquid as it is directed in a thin film past the zone the containers being filled with the treated liquid by direct connection with the interior of the vacuum chamber |
| G | 502 726 | April 3 1927 | H Geffcken and H Richter | Foods such as milk are subjected to ultraviolet rays |
| G | 523 257 | Feb 8 1927 | H Geffcken and H Richter Ass to A G f Elektricitäts Ind | Apparatus for irradiating food stuffs |
| G | 526 141 | May 17 1929 | H Geffcken and H Richter | Apparatus for irrad ating foods with ultraviolet rays |
| G | 530 877 | Nov 4 1928 | F Heinemann | Foodstuffs are vitaminized by ultra violet radiation using a calc spar filter |
| G | 545 080 | Feb 25 1932 | H Geffcken and H Richter | Irradiation of skimmed milk |
| G | 556 716 | Mar 7 1928 | Merck | Stable aqueous colloidal solutions of irradiated ergosterol are obtained by irradiating ergosterol in its dis persed form and separating the un changed ergosterol after irradiation |
| G<br>G | 564 401<br>608 277 | Nov 1 1927<br>Jan 19 1935 | F Kielwein | Apparatus for irradiating bread or yeast for incorporation into bread |
| G<br>G | 564 736<br>566 744 | Nov 22 1932<br>Dec 20 1932 | E Latacz | Apparatus for irradiation of liquids |
| G | 567 333 | Dec 31 1932 | Hoffmann LaRoche | Production of water soluble sterol compounds by irradiating sterols in the form of their ester salts *e g* a salt of phthal c acid monoergos terol ester with ultraviolet light |
| G<br>G | 568 900<br>577 170 | Jan 5 1933<br>Aug 10 1933 | K Hembd an l Vitamin Fab rik | Irradiation of extracts of high vis cosity |
| G | 572 491 | Mar 17 1933 | A Schindler | Ultraviolet irradiation of milk |
| G | 577 531 | June 1 1933 | ( Kersten and O K Schultz | Irradiation of skimmed milk fol lowed by addition of the original cream |
| G | 583 731 | Sept 9 1933 | I C | Production of an antirachitically active material by treating lumi sterol or its esters with ultraviolet light and method of crystallizing the antirachitic prodi ct a the di nitro benzoate |
| G | 622 373 | Nov 27 1935 | Leo-Werke | Vitamin containing cosmetics from wool fat by irrad ation with ultra violet light |
| G | 624 325 | Jan 17 1936 | H Heitan and Kuntze s Ver waltungs G | Vitamins are produced in malt beer by l ght from a Mg arc |
| G | 632 783 | July 13 1936 | Hanovia | Irrad ation of milk with mercury vapor lamp |
| G | 648 326 | July 29 1937 | E Freitag | Irradiation of m lk in bottles |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Conversion of Provitamins D to Vitamins D (*Continued*) | | | |
| G 673 852 | Mar 30 1939 | K Wolff and R Havemann | Method for obtaining transformation products of ergosterol and similar materials by irradiating with electrons from a glowing cathode in which the material to be activated is passed in thin layers over the anode |
| F 666 959 | Oct 8 1929 | Osa Part Ind Soc | Apparatus for the irradiation of liquids |
| F 667 660 | Oct 19 1929 | A Tribout | Pasteurization and irradiation of milk |
| F 677 010 | Mar 3 1930 | J Seip | Irradiation of beverages |
| F 677 111 | June 17 1929 | I G | Preparation of antirachitic substances by irradiation of provitamins at a temperature above 70 C |
| F 697 367 | Jan 16 1931 | T Reiter | Conversion of ergosterol to vitamin D by means of light of wave lengths longer than 280 mμ |
| F 700 036 | Nov 16 1929 | H Labbé | Sterols extracted from cacao beans *are activated by ultraviolet light* |
| F 708 548 | July 24 1931 | N V Philips Gloeilampenfabrieken | Irradiation of provitamins in solution in special apparatus |
| F 7 2 961 | Sept 20 1933 | J Major | Vitamin-containing flour by special treatment of grain and ultraviolet irradiation |
| F 779 847 | April 13 1935 | C Devret & Co | Irradiation of cholesterol-containing foods |
| F 8 1 4?1 | *Jan 9 1940* | *Hermes Patentverwertungs*-Gesellschaft | *Method and apparatus for irradia*ting milk sprays in a carbon dioxide atmosphere |
| Austrian 118 762 | Mar 15 1930 | O Ried | The irradiation effect is enhanced by the addition of mineral substances *e g* ZnO |
| Austrian 118 764 | Mar 15 1930 | O Ried | Enhancement of the biological effect of irradiated substances by the addition of trypaflavin |
| Austrian 137 455 | May 11 1934 | Leo Werke | Apparatus for irradiating wool fat or wool fat alcohols to enrich the vitamin D content |
| Belg 386 419 | Mar 31 1932 | Soc Rémiz | Vitamin-containing foods by irradiation while stirring |
| Hung 100 696 | Aug 4 1927 | G Feher | Yeast is saponified and extracted The isolated ergosterol is irradiated to form vitamin D |
| Hung 104 ?7 | April 14 1931 | A Jendrassik | Production of vitamin D from solid ergosterol in contact with a solution which dissolves the irradiated ergosterol and which solution is continuously removed |
| Vitamins D | | | |
| U S 1 563 134 | Nov 24 19 5 | T F Zucker Ass to University Patents | Process of assaying the antirachitic strength of a substance by determining the pH value of the feces of experimental animals. |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Vitamins D *(Continued)* | | | |
| U S 1 902 745 | Mar 21 1933 | A Windaus<br>Ass to Winthrop | The antirachitic vitamin obtained by ultraviolet irradiation of ergosterol is purified by the formation of addition products of the non vitamin material with compounds of the *maleic anhydride type* |
| U S 1 902 785<br>B 370 743<br>B 405 321<br>G 565 900<br>G 576 021 | Mar 21 1933<br>April 14 1932<br>Feb 5 1934<br>Dec 7 1932<br>May 6 1933 | O Linsert<br>Ass to Winthrop | Process which comprises freeing an antirachitically active product from unchanged ergosterol by subjecting the product to the action of at least one mole of maleic or citraconic acid anhydride |
| U S 2 030 377<br>G 603 088 | Feb 11 1936<br>Sept 22 1934 | O Linsert<br>Ass to Winthrop | Crystalline vitamin $D_2$ from irradiated ergosterol by formation of the 3 5-dinitrobenzoate followed by saponification |
| U S 2 099 550<br>B 491 653<br>G 661 686 | Nov 16 1937<br>Sept 6 1938<br>June 24 1938 | A Windaus and F Schenck<br>Ass to Winthrop | Process for the purification of vitamin $D_3$ obtained by activation of 7 dehydro-cholesterol by esterification with meta dinitro benzoyl chloride fractional crystallization of the esters formed and saponification of the meta dinitro benzoate of the vitamin $D_3$ Purificat on may also be accomplished by precipitation of 7 dehydro-cholesterol as the digitonide or by the formation of addition products with compounds *of the maleic anhydride type* |
| U S 2 179 560 | Nov 14 1939 | S E Miller<br>Ass to General Mills | Process of concentrating vitamin D from either naturally occurring or synthetic vitamin D concentrates by dissolving vitamin D containing material in an organic solvent passing the solution through tricalcium phosphate whereby a major portion of the vitamin D is adsorbed by the phosphate wash ng the phosphate with an organic solvent and then separating the vitamin D from the *solvent by distillation* |
| U S 2 216 719 | Oct 8 1940 | A G Boer J van Niekert A van Wijk and E H Reerink<br>Ass to Hartford National Bank & Trust Co | Process for producing a new chicken active vitamin D by activation of a new provitamin D derived from periwinkles |
| B 335 277<br>I 698 040 | Sept 25 1930<br>June 11 1930 | Société des Usines Chimiques Rhone Poulenc | Separation of ergosterol *from its* crude irradiation products by crystallization from an organic solvent particularly alcohol acetone and ethyl acetate |
| B 464 066<br>G 678 533<br>I 815 545 | April 12 1937<br>July 17 1939<br>July 13 1937 | N V Phil ps Gloeilampen fabrieken | Process of producing a preparation which has a h gh antirachit c activ ity for chickens wherein the un saponifiable fraction or the sterol fraction of duck eggs is irradiated with ultraviolet light The provitam n D is identical with 7 dehydro-cholesterol |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Vitamins D (*Continued*) | | | |
| B 482 880 | June 17 1937 | Eastman Kodak | Isolation of a new vitamin D by molecular dist llation of degassed vitamin D conta ning o ls |
| B 491 007 | Aug 23 1938 | Eastman Kodak | Esterificat on of vitamin D or of prov tamin D with unsaturated h gher fatty acids especially with linolen c acid The pro itam n D ester is activated to vit min D e ter |
| B 517 214 | Jan 24 1940 | Eastman Kodak | H gh vacuum short path distilla tion of natural vitamin D esters re sulting in the separation of 5 or 6 different antirachitic materials |
| G 550 496 | Apr 127 1930 | L Brauer and H Seel | Vitamins by oxidation of cholesterol with benzoyl peroxide |
| G 659 882 | May 17 1938 | H Brockmann Ass to I G | Isolation of the antirachitic vita min from natural products |
| G 672 000 | Feb 18 1939 | H Fincke Ass to Gebrüder Stollwerck | Fats with high vitamin D content are obtained by extracting ground cocoa shells with cocoa butter |
| Can 379 424 | Feb 9 1939 | L Yoder Ass to Iowa State College Alumni Assoc | Antirachitic substance by heating cholesterol with a mixture of concen trated sulfur c acid acet c anhy dride and acetic acid |
| Derivatives and Utilization | | | |
| U S 1 8?4 653<br>B 334 002 | Sept 22 1931<br>July 1 1929 | E Brauchl Ass to Hoffmann LaRoche | Irradiated ergosterol is stabilized by the addition of a small quantity of a dihydroxy phenol such as hydroquinone |
| U S 1 974 808 | Sept 25 1934 | C F Dietz Ass to Commander Larabee Corp | The oil from a germ bearing cereal grain is irradiated to form vitamin D and is incorporated into flour |
| U S 2 010 792 | Aug 6 1935 | J S egel Ass to A M Siloan | Collo dal d spers on of sterols in water containing bonemeal and al falfa extract |
| U S 2 0?2 464 | Nov 26 1935 | L A Hall Ass to C L Griffith | An emulsified vitamin D concen trate containing gum tragacanth and gum acacia the emulsion hav ing a pH of 5 5 to 6 0 |
| U S 2 030 792<br>B 436 713<br>B 469 150<br>G 64? 261 | Feb 11 1936<br>Oct 16 1935<br>July 20 1937<br>Feb 27 1937 | C W Hooper Ass to Winthrop | Propanediols and butanediols as solvents for fat soluble vitamins especially for vitamins D |
| U S 2 070 117<br>G 6?4,231 | Feb 9 1937<br>Jan 15 1936 | O Dalmer and F v Werder Ass to Winthrop | Hydrogenation of tachysterol-dini trobenzoate to form dihydro-tachy sterol |
| U S 2 150 316 | Mar 14 1939 | A E Briod and B R. East Ass to National Oil Products | Vitamin D milk is prepared by homogenizing a cod liver oil concen trate with cream or evaporated milk followed by canning and sterilization |
| U S 2 175 340 | Oct 10 1939 | J W D Chesney Ass to New Discoveries Inc | A vitamin D concentrate is added to a prod ct such as beer contain ing at least 0.25% of alcohol and dissolved $CO_2$ |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Derivatives and Utilization** (*Continued*) | | | |
| U S 2 18_ 969 | Jan 2 1940 | H E Schultze<br>Ass to Winthrop | Preparation of clear aqueous thera peutic solution of vitamin D with triolein polyethylene glycol oleyl ether and physiological NaCl solu tion |
| U S 2 194 188 | Mar 19 1940 | G C Supplee<br>Ass to The Borden Co | Method for producing a vitamin D protein symplex of enhanced anti rachitic activity |
| U S 2 228 491 | Jan 14 1941 | F v Werder<br>Ass to Winthrop | Isolation of pure dihydro-tachy sterol |
| U S 2 245 418 | June 10 1941 | R C Sherwood and C G Ferrari<br>Ass to General Mills | Production of a sterile emulsion of evaporated milk fortified with a concentrate of vitamin $D_2$ by dis solving activated ergosterol in but ter fat and then dispersing the butter fat concentrate in evapor ated milk and canning and steriliz ing the resulting product |
| U S 2 264 320 | Dec 2 1941 | O Linsert<br>Ass to Alba | Vitamin $D_2$ double compounds with cholesterol cholestanol or copro- sterol |
| U S 2 265 320 | Dec 9 1941 | R C Sherwood and C G Ferrari<br>Ass to General Mills | Emulsion of vitamin $D_2$ in evapo- rated milk |
| B 40_ 791 | Feb 15 1934 | *Bell and Sons Ltd and J Sow ler* | *Vitamin D containing oils are* ab sorbed in flour or other carriers of porous nature and mixed with min eral matter for animal feeds |
| B 406 629<br>F 737 234 | Not accepted<br>May 17 1932 | N V Philips Gloeilampen fabrieken | Vitamin D is preserved by adding oil or fat to a solution of ergosterol before during or immediately after irradiation in the absence of oxygen |
| B 412 53_ | July 11 1934 | N V Philips Gloeilampen fabrieken | Poultry feeds containing vitamin $D_2$ |
| B 449 888 | April 13 1935 | Standard Brands | Increase in vitamin D content of *eggs by feeding hens vitam n D* |
| G 495 4_0 | June 30 1927 | Hoffmann LaRoche | Sterols other than cholesterol *e g* ergosterol are w ter *solubilized by* the formation of mono esters of dicarboxylic acids |
| G 501 9_4<br>Add to<br>49_ 450 | Aug 6 1927 | Hoffmann LaRoche | Photo activated ergosterol is water solubilized by the formation of mono-esters of dicarboxylic ac ds |

## VITAMINS E

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|---|---|---|---|
| **Isolation** | | | |
| U S 2 188 878<br>G 6_1 474 | Jan 30 1940<br>Oct 15 1937 | C L Lautenschläger and F Lindner<br>*Ass to W nthrop* | Process for preparing *crystallized* vitamin E allophanates by esteri fication with cyanuric acid followed by purification by adsorption on alum num oxide followed by elu tion |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Isolation (*Continued*) | | | |
| U S 2 203 400<br>B 531 2 4 | June 4 1940<br>Dec 31 1940 | J S Andrews<br>Ass to General Mills | Vitamin E concentr te from wheat germ oil by catalytically hydrogenating the oil extracting the hydrogenated oil w th alcohol and further concentrating by separating the sterols and glycerides at low temperatures saponifying and extracting |
| U S 2,263 550 | Nov 18 1941 | J S Andrews<br>Ass to General Mills | Preparation of vitamin E concentrate from wheat germ oils by esterification with a monohydric alcohol followed by distillation of the vitamin E ester |
| B 31 ??0 | Dec 31 1940 | General Mills | Process for preparing a concentrate of vitamin E from vegetable oils by ester interchange of the glycerol esters with a lower aliphatic monohydric alcohol to yield glycerol and esters of monohydric alcohols followed by separating the glycerol and the sterols and saponifying the remaining mixture |
| Synthesis | | | |
| U S 2 208 585 | July 23 1940 | P Karrer<br>Ass to Hoffmann LaRoche | Synthesis of vitamin E by condensation of alkylated hydroquinones with halogen derivatives of phytol |
| U S 2 215 398<br>B 528 371 | Sept 17 1941<br>Oct 28 1940 | P Karrer<br>Ass to Hoffmann LaRoche | Separation of alpha tocopherol from its racemic synthetic mixture comprising the treatment with 3 bromocamphor sulphonic ac d chloride isolation of the condensation product and hydrolysis to yield the free pure alpha tocopherol |
| U S 2 30 659 | Feb 4 1941 | F v Werder<br>Ass to Merck | Process for the production of alpha tocopherol comprising condensing trimethyl hydroquinone with phytol in the pre nce of phosphorus pentoxide |
| U S 2 245 147 | June 10 1941 | W John and P Günther<br>Ass to Merck | Synthe is of compounds of the general form la of v tamin E by react i g a d hydro coumarin with a mixture of methyl magne um h logen de and the Grignard compound from a higher halogen ted hydrocarbon |
| U S 2,249 054<br>B 29 082<br>(Add to<br>B 529 081) | July 15 1941<br>Nov 13 1940 | L I Smith and H E Ungnade<br>Ass to University of M nnesota | Manufacture of tocopherol like compoun ds by re cting hydroquinones or their monoethers w th d enes in the pre ence of an ac dic substance |
| B 0 884 | Sept 27 1940 | Hoffmann LaRoche | Process for the manufacture of *dl* tocopherol n which methyl substituted hydroquinones are reacted with phytol isophytol phytyl hal de or 3 halogeno-d hydro-phytyl h l des in the presence of z nc chlor de or form c acid |
| B ?7,3?0 | Oct 8 1940 | Hoffmann LaRoche | Cond nsat on of trimethyl hydroquinones and alkyl ne hal des (with the e ception of phytyl halides) |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| **Synthesis** (*Continued*) | | | |
| B 528 372 | Oct 28 1940 | Hoffmann LaRoche | Condensation of alkyl hydroquinones with halides of alpha beta unsaturated carboxylic acids in the presence of an acid condensing agent |
| B 529 081<br>F 855 414 | Nov 13 1940<br>May 10 1940 | University of Minnesota | Manufacture of coumarins and chromanes by condensation of hydroquinones alkylated or not alkylated with substituted or unsubstituted alkyl halides |
| B 532 364 | Jan 22 1941 | Hoffmann LaRoche | Acetyl *dl* alpha tocopherol by acetylation of *dl* alpha tocopherol |
| B 537 774 | July 7 1941 | Hoffmann LaRoche | Manufacture of tocols from phytyl halides and alkylated hydroquinones |
| B 539 097 | Sept 29 1941 | Hoffmann LaRoche | Synthesis of vitamin E from trimethyl hydroquinone *via* the mono-alkylene ether (*e g* of phytol) by intramolecular condensation |
| B 540 907 | Nov 5 1941 | Hoffmann LaRoche | Synthesis of ethers and esters of tocols by condensation of phytol compounds with mono ethers or mono-esters of dimethyl hydroquinones |
| B 541 008 | Nov 10 1941 | Hoffmann LaRoche | Manufacture of ring homologues of vitamin E by condensation of phytol compounds with alkylated hydroquinones |
| B 541 011 | Nov 10 1941 | Hoffmann LaRoche and J F Pollak | Synthesis of vitamin E by rearrangement of trimethyl hydroquinone mono-phytyl ether followed by condensation under acidic conditions |
| G 374 142 | April 20 1923 | L Claisen | Synthesis of chromanes by condensation of phenols with butadiene hydrocarbons in the presence of acidic condensing agents |
| G 703 957 | Mar 20 1941 | O Hromatka<br>Ass to Merck | Synthesis of vitamin E by condensation of 3 amino-6 oxy 1 2 4 trimethyl benzene with phytol compounds followed by conversion of the amino into a hydroxyl group |
| **Intermediates for the Synthesis** | | | |
| U S 2 229 573<br>C 683 908<br>Add to<br>C 676 198 | Jan 21 1941<br>Nov 18 1939 | F Jung<br>Ass to Merck | Trimethyl hydroquinone from trimethyl quinone by catalytic hydrogenation |
| U S 2 229 574 | Jan 21 1941 | F Jung<br>Ass to Merck | Xylohydroquinone by catalytic hydrogenation of xyloquinone |
| U S 2 259 936<br>G 676 198 | Oct 21 1941<br>May 30 1939 | F Jung<br>Ass to Merck | Duro-hydroquinone from duroquinone by catalytic hydrogenation |
| B 508 292 | June 28 1939 | Hoffmann LaRoche | Manufactur of phytyl bromide |
| B 537 793 | July 7 1941 | Hoffmann LaRoche | Condensation of trimethyl hydroquinone with acetyl phytol to yield phytyl trimethyl o mono acetyl hydroquinone |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| Derivatives and Utilization | | | |
| U S 2 167 002 | July 25 1939 | A. J Pac m<br>Ass to U S Vitam n Corp | Composition of matter claims for vitamin E and magnesium d strib uted therein in an amount not less than 0 05% |
| U S 2,212 531 | Aug 27 1940 | F v Werder<br>Ass to Merck | Process claims for the reaction of durohydroquinone w th long chain alkyl halides alkylene h l des and hydro aromatic halid s to form compounds of the type $C_6(CH\ )(OH)OR$ R = 6–14 car bons Product claims for the lat ter compounds w th vitamin E activ ty |
| U S 2 21? 532 | Aug 27 1940 | F v Werder<br>Ass to Merck | Process cla ms for the react on of trimethyl hydroqu none with long cha n alkyl hal des alkyene hal des and hydro aromatic halides to form compounds of the type $C_6H(CH\ )(OH)OR$ R = 6 14 c r bon atoms Product claims for the latter compounds w th itamin E acti ty |
| U S 2 ?16 841<br>B 506 569<br>B 527 006 | Oct 8 1940<br>May 31 1939<br>Sept 30 1940 | O Isler<br>Ass to Hoffmann LaRoche | Composition of matter cla ms to duro hydroqu none mono phy tyl ether and process for prepara t on by reacting durol y droqu non w th a phytyl hal de in the presenc of an alkali metal carl onate |
| U S 2 ?31 125<br>B 536 60? | Feb 11 1941<br>May 21 1941 | P Karrer<br>Ass to Hoffmann LaRoche | Comp s t on of matter cla m for tocopherol-oleate and stearate |
| U S ? 35 884 | Mar 25 1941 | W John and O Dalmer<br>Ass to M rck | Proces for the manufact re of monoethers of tr methyl hydroqui none by treatment with aliphatic or aromatic aturated or un aturated lcohols ter or hal des Product cla ms for the octadecyl and no decyl mono-ethers. |
| U S 2 245 480<br>B 5?9 023<br>G 702 491 | June 10 1941<br>Nov 12 1940<br>Feb 8 1941 | P Karrer<br>Ass to Hoffmann LaRoche | Process for the manufacture of con densation products from beta toco-pherol and alpha beta unsatu rated alkyl hal des |
| U S 2 247 364<br>G 704 171 | July 1 1941<br>Mar .5 1941 | E Fernholz<br>Ass to Merck | Mono and d alkyl eth rs of d iro hydroquinone |
| B 517 932 | Feb 13 1940 | Ciba | Manufacture of mono ethers from alkylated para d hydro y benzenes and al phatic alcohols containing 10 or 11 carbon atoms forming a branched carbon ch in to which may also be l nked an al cycl c resi due Also esterification of the free hydroxyl group of the react on product |

VITAMIN H

| | | | |
|---|---|---|---|
| U S 2 193 5 3 | Mar 12 1940 | F Schultz<br>Ass to W nthrop | Vitam n H is l berated from ti sue protein by hydrolysis at elevated temper tures and extraction with org n c sol ents |

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 02 307 | May 28 1940 | L E Booher | Concentration of vitamin H by adsorption of an alcoholic solution on activated carbon followed by elution with alcohols benzene alcohol or acetone |
| B 459 524<br>G 645 414 | Jan 11 1937<br>May 27 1937 | I G | Purification of vitamin H preparations by electrodialysis |
| B 403 698<br>G 664 088<br>G 661 435 | April 5 1937<br>Aug 19 1938<br>Oct 15 1937 | F Schultz<br>Ass to I G | Extraction of vitamin H from animal organs by energetic hydrolysis followed by acetone addition which precipitates impurities and leaves the vitamin in solution |
| B 484 981<br>F 824 878 | May 9 1938<br>Feb 17 1938 | I G | Treatment of seeds with biotin or derivatives in various carriers |
| G 661 929 | June 30 1938 | I G | Biotin extracts from yeast seeds germs egg yolk etc by precipitation of impurities in crude extracts by means of Reinecke salt p crolonic or oxalic acid or by acetylation Precipitation of biotin with phosphotungstic acid or mercuric chloride |
| G 670 098 | Jan 11 1939 | I G | Precipitation of biotin with $H_2PtCl_6$ |
| G 670 922<br>Continuation of 670 098 | Jan 27 1939 | I G | Isolation of biotin after previous esterification |

## VITAMINS K

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 233 279 | Feb 25 1941 | S Ansbacher E Fernholz and M L Moore<br>Ass to Squibb | Process for concentrating vitamin K by adsorption on activated charcoal followed by elution |
| B 533 513 | Feb 14 1941 | St Louis University | Isolation of vitamins K by adsorption on a base exchange silicate *e g* sodium aluminum silicate followed by extraction with non polar solvents *e g* benzene |
| B 539 471 | Sept 11 1941 | Parke Davis | Manufacture of 2 methyl 4 amino naphthol from 1 4 naphthoquinone or nitro-methyl naphthalene |
| B 541 138 | Nov 13 1941 | Hoffmann LaRoche | Manufacture of acid succinic acid esters of alkylated 1 4 naphthohydroquinones |

## VITAMIN P

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 2 224 807 | Dec 10 1940 | M Brockmühl and E Bartholomaus<br>Ass to Winthrop | Process for the production of glucosides of polyhydroxy flavanones |
| B 486 898<br>Can 398 341 | June 13 1938<br>July 29 1941 | A Szent Györgyi<br>Ass to Winthrop | Process for the extraction of flavones from vegetable material by means of an organic solvent miscible with water precipitation with an alkali or earth alkali metal hydroxide followed by acid decomposition |

CHOLINE

| Patent No | Date | Patentee | Abstract |
|---|---|---|---|
| U S 1 904 00<br>B 315 713 | April 18 1933<br>Jan 13 1930 | G Roy<br>A s to l e Rhône Poul nc | Solution of acetyl-ch line salts in ethylene glycol monoacetate or glyc rol mono- or 1 acetate |
| U S 1 9 7 443 | M y 8 1934 | J K Cl ie<br>Ass to Merck | Salt of acetyl ted cholines |
| U S 1 957 401 | May 8 1934 | L W Jones and R T Major<br>Ass to Merck | Acetyl chol ne alts |
| U S 2 043 403 | A g 4 1936 | R T Major and J Kl ne<br>Ass to Merck | Salts of beta alkylated chol ne alkyl ethers |
| B 8 031/14 | Mar 30 1914 | J Y Johnson | S lt of choline |
| B 379 00<br>F 736 107 | Aug 25 1932<br>April 9 1932 | F Körner | Prep rat on of alts of chol ne |
| G 290 3 | Aug 12 1913 | Hoffmann LaR ch | Non hyg oscop c salts of chol ne w th 1 halohydroxybenzo c acids |
| G 30 7 | May 1 1918 | Chem W e ke Crenzach | Arson um compounds of the chol ne type |
| G 590 311 | Jan 10 1934 | M rck | Quaternary salts of choline deriva tives |
| G 33 258 | Feb 23 1934 | L Glucksmann | Chol ne salts of b le ac ds |
| G 638 041 | Nov 20 1936 | Hoffmann LaRoche | Alcohol c solutions of hygroscopic salts of acetylcholine |

RECOMMENDED DIETARY ALLOWANCES[a]

Recommended by the Food and Nutrition Board National Research Council

| Group | Calories | Protein g | Calcium g | Iron mg | Vitamin A[c] I U | Thiamin ($B_1$) mg[b] | Riboflavin mg | Niacin (nicotinic acid) mg | Ascorbic acid mg[b] | Vitamin D I U |
|---|---|---|---|---|---|---|---|---|---|---|
| Man (70 kg) | | | | | | | | | | |
| Sedentary | 2500 | | | | | 1 5 | 2 2 | 15 | | |
| Moderately active | 3000 | 70 | 0 8 | 12 | 5000 | 1 8 | 2 7 | 18 | 75 | |
| Very active | 4500 | | | | | 2 3 | 3 3 | 23 | | |
| Woman (56 kg) | | | | | | | | | | |
| Sedentary | 2100 | | | | | 1 2 | 1 8 | 12 | | |
| Moderately active | 2500 | 60 | 0 8 | 12 | 5000 | 1 5 | 2 2 | 15 | 70 | |
| Very active | 3000 | | | | | 1 8 | 2 7 | 18 | | |
| Pregnancy (latter half) | 2500 | 85 | 1 5 | 15 | 6000 | 1 8 | 2 5 | 18 | 100 | 400–800 |
| Lactation | 3000 | 100 | 2 0 | 15 | 8000 | 2 3 | 3 0 | 23 | 150 | 400–800 |
| Children up to 12 years | | | | | | | | | | |
| Under 1 yr[d] | 100/kg | 3 4/kg | 1 0 | 6 | 1500 | 0 4 | 0 6 | 4 | 30 | 400–800 |
| 1–3 yrs[e] | 1200 | 40 | 1 0 | 7 | 2000 | 0 6 | 0 9 | 6 | 35 | [f] |
| 4–6 yrs | 1600 | 50 | 1 0 | 8 | 2500 | 0 8 | 1 2 | 8 | 50 | |
| 7–9 yrs | 2000 | 60 | 1 0 | 10 | 3500 | 1 0 | 1 5 | 10 | 60 | |
| 10–12 yrs | 2500 | 70 | 1 2 | 12 | 4500 | 1 2 | 1 8 | 12 | 75 | |
| Children over 12 years | | | | | | | | | | |
| Girls 13–15 yrs | 2800 | 80 | 1 3 | 15 | 5000 | 1 4 | 2 0 | 14 | 80 | |
| 16–20 yrs | 2400 | 75 | 1 0 | 15 | 5000 | 1 2 | 1 8 | 12 | 80 | |
| Boys 13–15 yrs | 3200 | 85 | 1 4 | 15 | 5000 | 1 6 | 2 4 | 16 | 90 | [f] |
| 16–20 yrs | 3800 | 100 | 1 4 | 15 | 6000 | 2 0 | 3 0 | 20 | 100 | |

**Footnotes to Table**

[a] Tentative goal toward which to aim in planning practical dietaries can be met by a good diet of natural foods Such a diet will also provide other minerals and vitamins the requirements for which are less well known

[b] 1 mg thiamin equals 333 I U 1 mg ascorbic acid equals 20 I U

[c] Requirements may be less if provided as vitamin A greater if provided chiefly as the provitamin carotene

[d] Needs of infants increase from month to month The amounts given are for approximately 6–8 months , The amounts of protein and calcium needed are less if derived from human milk

[e] Allowances are based on needs for the middle year in each group (as 2 5 8 etc) and for moderate activity

[f] Vitamin D is undoubtedly necessary for older children and adults When not available from sunshine it should be provided probably up to the minimum amounts recommended for infants

*Further Recommendations Adopted 1942*

*The requirement for iodine is small probably about 0 002 to 0 004 milligram* a day for each kilogram of body weight This amounts to about 0 15 to 0 30 milligram daily for the adult This need is easily met by the regular use of iodized salt its use is especially important in adolescence and pregnancy

The requirement for *copper* for adults is in the neighborhood of 1 0 to 2 0 milligrams a day Infants and children require approximately 0 05 per kilogram of body weight The requirement for copper is approximately one tenth of that for iron

The requirement for *vitamin K* is usually satisfied by any good diet Special consideration needs to be given to newborn infants Physicians commonly give vitamin K either to the mother before delivery or to the infant immediately after birth

# AUTHOR INDEX*



* It he n meral r f r to th l t t l ide

D

## F

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## J

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## S

T

## U

# SUBJECT INDEX

[ABBREVIATIONS acty (activity) defn (definition) defcy (deficiency) dtn (determination) dehydn (dehydrogenation) hydn (hydrogenation) oxdn (oxidation) provit (provitamin) vit (vitamin) ]

B

## C

E

F

## M

## N



## O



## P

## U